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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—FARNUM.

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CHEMICAL CRITERIA OF ANAEROBIOSIS WITH SPECIAL REFERENCE TO METHYLENE BLUE[1]

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From the Department of Hygiene and Bacteriology, University of Chicago

Received for publication June 4, 1920

The literature of anaerobic technology contains frequent references to various criteria of anaerobiosis aside from growth of organisms. To be sure, the successful cultivation of a known anaerobic micro-organism under given conditions, in contrast with the failure of growth of the same organism on the surface of solid media of similar composition in free contact with air, constitutes a satisfactory *biological* criterion of anaerobiosis for the particular organism used in the test and under the special conditions thereof. But there are circumstances in which it is desirable to correlate other means of determining oxygen tension reduction. An obviously useful *physical* means is the vacuum manometer, but most helpful of all are *chemical* criteria, which are usually based upon coloration changes.

One of the earliest indicators used, and a notable exception to the rule of indicators with coloration changes, was phosphorus, whose failure to ignite was employed by Gratama, a student of Gunning's (1877).

A mixture of alkali with pyrogallic acid, as used in reducing oxygen tension for the cultivation of anaerobic organisms, is also frequently mentioned as affording a criterion of successful anaerobiosis since in the absence of oxygen the solution remains nearly or quite colorless. But it is scarcely possible for this

[1] This essay is based upon an investigation completed during the writer's tenure of the Logan Fellowship at the University of Chicago and is one of a series awarded the Howard Taylor Ricketts Memorial Prize for 1920.

reagent to serve both as a means of removing oxygen and as a criterion of removal at the same time, so that the latter purpose can be achieved only when combined with other means of oxygen tension reduction.

Fermi and Bassu (1904), using alkaline pyrogallol as a criterion, encountered extreme difficulty in demonstrating complete anaerobiosis. For example, they found that boiling media under paraffine oil for over one hour does not prevent the darkening of an alkali-pyrogallol mixture placed therein and a similar statement was made respecting the passage of hydrogen and carbon dioxide through media. It appears from my own experiments that the colored compounds formed by the action of oxygen upon alkali-pyrogallol mixtures are quite stable and the reactions irreversible, for neither very weakly alkaline-solutions which show only a trace of color with pyrogallic acid nor strongly alkaline deep colored solutions can be decolorized by prolonged boiling. Therefore the difficulty of making the mixture without obtaining some coloration and the further impossibility of removing it previous to actual test distinctly limit the practicability of alkaline pyrogallol as a criterion of anaerobiosis, notwithstanding its great value as a means of oxygen tension reduction.

More extensive use has been made of substances which in the absence of free oxygen are reduced to leucobases. Some of these can be used, not only in media during the active growth of organisms, but separately as well, for estimating the suitability of special apparatus. Among such indicators may be mentioned potassium ferro-ferro cyanid, litmus, indigo (sodium indigo sulphonate) and methylene blue.

Potassium ferro-ferro cyanid [$(K_2Fe\ (Fe\ Cy_6))$] is of slight historical, but no practical, importance. It was used by Gunning (1877) (1878) (1879) and is said to become colorless [$Fe_2Fe\ Cy_6$] when air is eliminated.

The earliest authentic reference to the bacteriological use of litmus appears to be that of Würtz (1892) who introduced litmus lactose agar as a differential medium for *Bact. coli* and *Bact. typhosum*. It was impossible to confirm Novy's (1893) allusion [copied by Hunziker (1902)] to Buchner (1885) and Cohen (?)

as first to use litmus to indicate acid and reduction changes respectively, the last reference apparently being altogether erroneous. The decolorization of indigo and methylene blue in culture media were studied by Spina (1887) whose interest in these dyes hinged rather upon their reduction by bacterial growth though he recognized the phenomenon as occurring most vigorously in the depths and noted the return of color on exposure to the air. Kitasato and Weyl (1890) confirmed this observation so far as regards sodium indigo sulphate. The decolorization of all three dyes by sterile culture media under anaerobic conditions, as well as by living aerobic and anaerobic cultures, was especially investigated by Smith (1893) (1896) who noted the necessity of some organic substance such as glucose or peptone and an alkaline reaction in the case of sterile media decolorized by heat.

I found neutral litmus solutions unaffected in color by heating for twenty minutes in a boiling water bath, and the same is true of litmus with 1 per cent glucose. Litmus solutions with 1 per cent glucose and HCl stronger than N/8 were precipitated by heating and the precipitate was not redissolved on cooling; weaker acid solutions were unaffected except for reddening. Strong alkali N/2 to N/32 caramelized the sugar and decolorized the dye permanently; weaker solutions decolorized on boiling for a few minutes and regained their original blue color only on exposure to air.

The recoloration of such decolorized solution of litmus, indigo and methylene blue by exposure to air indicates reversible reactions and constitutes the key to the use of such dyes as criteria of anaerobiosis.

McLeod (1913) cleverly utilized the blue laboratory pencil mark as a criterion of anaerobiosis upon the basis of its decolorization in the absence of air. Some pencils fail to respond, however, according to my experience.

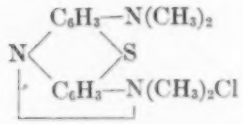
METHYLENE BLUE AS A CRITERION OF ANAEROBIOSIS

The most valuable and most extensively advocated chemical criterion of anaerobiosis is methylene blue. Introduced as an ingredient of culture media by Spina (1887), studied as an indi-

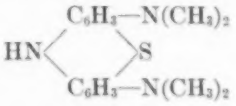
cator of anaerobiosis by Smith (1893) and others, it has been used to a certain extent by almost every serious investigator of anaerobiosis since. Smith (1893) (1896) noted its decolorization in the closed arm of the fermentation tube. Sanfelice (1893) and Liefmann (1908) defended the use of glass slips on the basis of the decolorization of methylene blue in the underlying agar. Trenkmann (1898) and Rivas (1902) used it in their culture tests with Na_2S as a reducing agent. Kabrhel (1899) used it in his bell jar device for plates and thereby showed the necessity of removing the covers for efficacious absorption of oxygen by alkaline pyrogallol; he also showed its value as an indicator of the anaerobiosis of deep culture media, liquid and solid. It was used by Petri (1900) in connection with oxygen tension reduction by hydrogen and alkaline pyrogallol, by Sellards (1904) with phosphorus, by Fremlin (1903) (1904), Stüler (1904), Berner (1904), Lentz (1910) with various plating devices, by Wrzosek (1907) (1909), Liefmann (1907), Hata (1908), Guillemot and Szczawinska (1908), Zinsser, Hopkins and Gilbert (1915) with plant and animal tissues, by Laidlaw (1915), and McIntosh and Fildes (1916) in the use of spongy platinum and palladium black as hydrogen-oxygen catalysers, by Wilson (1917) in the use of coal gas, by Douglas, Fleming, and Colebrook (1917) in connection with many porous substances and by a great many others.

CHEMISTRY OF METHYLENE BLUE

Discovered by Caro in 1876 and used empirically for many years in the arts, methylene blue, as a chemical compound, was studied most authoritatively by Bernthsen. He showed (1883) that $Na_2S_2O_4$ reduces it to its colorless leuco base, methylene white, which may be crystallized out of ether and whose aqueous solution becomes dark blue again in acid solution with iron chloride. Möhlau (1883) expressed the rule that methylene white is changed to methylene blue by oxidizing agents in acid solution. Bernthsen (1883) engaged in a brief polemic with Möhlau (1883) and Erlenmeyer (1883) as to the structural formulae of these compounds and finally (1884) set down methylene blue chloride as

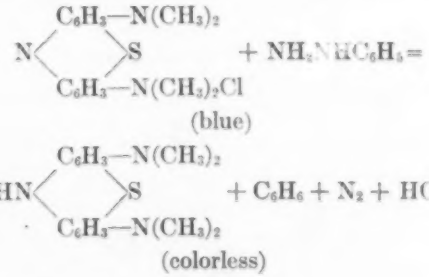


which is reduced (Bernthsen 1885) by the action of zinc or zinc chloride with HCl or H_2SO_4 and in alkaline solution with ammonium sulfate to leuco-methylene blue



These formulae are generally accepted now, practically the only disagreement being as to the direct bond between two of the nitrogen atoms.

Landauer and Weil (1910) also obtained leuco-methylene blue by treating a solution of the blue salt in alcohol, with phenylhydrazine, warming and cooling under CO_2. It has a melting point of 185°C. and is not oxidized even by pure oxygen in an atmosphere free from acid and in strongly alkaline solutions is not acted upon by permanganate or hydrogen peroxide. The following equation represents the reaction:



Excepting Landauer and Weil (1910) chemists have studied methylene blue largely from the standpoint of action of inorganic compounds upon it. We turn now to a consideration of its behavior in the presence of those factors which enter into bacteriological culture media, since the custom, not altogether defensible,

as I shall show, has grown up of adding a trace of this dye to the culture medium—either with or without inoculation—as a criterion of anaerobiosis.

We have already referred to the fundamental observations of Smith (1893) (1896) on the decolorization of methylene blue in alkaline solutions containing glucose or peptone under anaerobic conditions induced by heating. Kabrhel (1899) and Hammerl (1901) used such a solution along with their cultures as an indication of the successful exclusion of oxygen and the latter showed that the sugar might be replaced with sodium formate. Fremlin (1904) found an alkalinized methyl alcohol solution of methylene blue more delicate than an aqueous solution but recognized the possible inhibitory action of the volatile spirit upon bacterial growth.

As Bernthsen has shown, commercial methylene blue is likely to be a mixture with methylene azure, the latter being formed by the action of alkalis. Underhill and Closson (1905) have given methods for the purification of both, which however is not necessary in using methylene blue as a criterion of anaerobiosis since both compounds yield colorless leuco-bases under similar conditions; furthermore methylene azure is formed from methylene blue under conditions of alkalinity such as obtain in the test.

EXPERIMENTAL WORK

Decolorization—Preliminary discussion

The writer's interest in methylene blue as a criterion of anaerobiosis dates from the invention of the constricted tube and marble device (Hall, 1915). It was possible to show by its use that certain shipments of tubes contained 4 per cent with defective bore so that they could not be used. A properly made tube containing a methylene blue solution of certain composition, with a good marble seal will not permit the return of color below the marble for several days after decolorization by heating. One must not fall into the error of assuming, however, that decolorization of methylene blue necessarily indicates suitability for anaerobic growth; there are many factors, aside from the reduc-

tion of oxygen tension, in the cultivation of anaerobes. However, the failure of a *properly balanced* solution to remain decolorized indicates a defect in the method of air exclusion proposed. Decolorization of methylene blue probably occurs at a definite point during the abstraction of oxygen—a point yet to be determined. So decolorization may indicate suitability for some organisms and not for others. Methylene blue tests with McLeod's (1913) plate were satisfactory yet the bacteriological use of this plate in our hands was never satisfactory. Therefore, while we must admit that the decolorization of methylene blue solution, delicately adjusted, frequently correlates with successful anaerobic cultures, it is more important to recognize the limits and conditions of this test and to appreciate that the factors which enter into the successful decolorization of methylene blue are not necessarily common to the growth of all obligative anaerobes. It should be emphasized especially that acidification, probably through absorption of carbon dioxide from the air, may account for a return of color to decolorized methylene blue solutions and that in this case the dye cannot be bleached again without re-alkalinization.

Essential factors in decolorization

Preliminary experiments had to do with tests of Grübler's methylene blau für Bacillen in two culture media commonly used for the cultivation of anaerobes—magnesium carbonate glucose broth (Hall, 1915) and neutral (phenolphthalein) glucose agar. A trace of methylene blue in either of these media is easily decolorized by heating in a boiling water bath. In the open air such decolorized solutions quickly recover their original blue color but protected from air remain decolorized indefinitely. Thus in the constricted tube filled with glucose broth the color returns above but not below the marble seal; in deep glucose agar the color returns to the upper layers first and gradually descends. But it was noticed in certain cases of methylene blue glucose broth allowed to stand for several days that heating failed to decolorize the dye although it had done so originally. The outcome was a series of experiments to determine the

principal factors of decolorization and return of color in methylene blue solution.

Considering each of the ingredients of magnesium carbonate glucose broth as possible single factors in the decolorization of the dye, it was first shown that only those solutions slightly alkalinized, as by means of magnesium carbonate or sodium or potassium hydroxide, lose color on boiling. The use of magnesium carbonate referred to involves addition of an excess and the removal of the undissolved residue by filtration after boiling: only a trace of magnesium goes into solution and the reaction is made faintly alkaline (pH = about 8). With such adjustment it was found possible to dispense with the salt and any two of the other three factors (meat infusion, peptone, and glucose) without interfering with decolorization. But the clearest cut results were obtained with glucose present. Furthermore, rather prolonged boiling is required for decolorization if the glucose be added to the filtrate from a heated $MgCO_3$ suspension in water, i.e., is not heated in the presence of an excess of $MgCO_3$; a slightly alkaline solution of 2 per cent agar was also decolorized easily. Further experiments were then undertaken to determine the effect of variation in reaction upon methylene blue solutions in the presence of these various organic substances.

The following facts stand out as a result of many experiments.

Neutral aqueous solutions of Grübler's Methylene blau für Bacillen containing 0.0001 gram or more per cubic centimeter are not decolorized in a water bath boiling hard for twenty minutes. Neither the inorganic acids, HCl, H_2SO_4, HNO_3, nor the organic acids, oxalic, acetic, lactic, citric, butyric, succinic, formic, and propionic, in a concentration of N/10, have any visible effect when heated in weak solutions of methylene blue. Yet methylene blue is decolorized slowly in a solution of HCl acting on zinc in the presence of platinum. N/10 NH_4OH has no visible effect while the equivalent concentrations of BaOH, NaOH and KOH produce a violet lavender color only—indicating, no doubt, the formation of methylene azure.

Neutral glucose solutions ranging from 1 to 10 per cent and faintly or deeply colored with methylene blue are not decolorized

during thirty minutes in a boiling water bath. The same is true of 2 per cent Witte's peptone solutions and of 2 per cent agar solutions. Neither does the addition of 0.5 per cent glucose to any of the acid solutions mentioned above result in decolorization on heating; likewise 2 per cent agar and 2 per cent Witte's peptone in graded hydrochloric acid solutions up to N/10 for agar (which fails to solidify), and up to N/2 for peptone, refuse to decolorize on heating. The neutral sodium salts of the above acids formed by adding equivalent amounts of standardized NaOH do not alter the result; none decolorize on heating.

On the other hand an extremely small excess of alkali causes the heated glucose, agar or peptone solution of methylene blue to lose its color completely. Even such traces of alkali as may be dissolved from the glassware may cause the decolorization of methylene blue in glucose solutions on heating. Incidentally we recall that Laird (1913) found the reaction time for Fehling's solution reduced by boiling glucose, laevulose, galactose, maltose and lactose in various makes of German glassware, owing to the abstraction of calcium hydroxide from the glass. All experiments reported herein were made with glassware carefully cleaned with chromic acid cleaning fluid and rinsed in distilled water. The use of such weak concentrations of alkali, which were approximated only by dilutions of standardized N/1 solutions, involves the possibility of other factors of error, as for example, atmospheric CO_2 and non-neutral distilled water, which do not enter so fully with higher concentrations. Repeated tests of the distilled water by colorimetric tests with phenolsulphonephthalein showed the limits of pH value to be 6.8 and 7.0; thus this possible factor of error was excluded. The CO_2 factor of error was reduced as far as possible by the use of freshly boiled distilled water for the preparation of solutions and checked as a disturbing factor in the interpretation of results. The great difficulty in adequately and exactly controlling the very slight alkalinity of the solutions in different experiments without the use of buffer substances may account for some nonsignificant discrepancies between the results with high dilutions of alkali in different tests. It should be made quite clear that decolorization of

alkaline methylene blue solutions in the presence of these certain organic ingredients of culture media is quite independent of the presence or absence of the acid ions mentioned.

Neutral and N/20 HCl solutions of 1 per cent levulose, glucose, lactose, maltose, sucrose, raffinose, inulin, dextrin, mannitol and, dulcitol, (all Merck's highest purity), with 0.00005 gram methylene blue were tested also for decolorization by heating in a boiling water bath for ten minutes, with negative results. Glucose,

TABLE 1

Correlation of Fehling's test and decolorization of methylene blue by alkalinized sugar solution

	N/3NaOH	N/100NaOH	N/1000NaOH	NEUTRAL	N/20HCl	REDUCTION IN FEHLING'S TEST*
Levulose		+	+	−	−	+
Glucose		+	+	−	−	+
Lactose		+	+	−	−	+
Maltose		+	+	−	−	+
Sucrose	−	−	−	−	−	−
Raffinose		+	−	−	−	−
Inulin		+	−	−	−	?
Dextrin		+	−	−	−	?
Mannitol	−	−	−	−	−	
Dulcitol	−	−	−	−	−	

* Quoted from Hawk-Practical Physiological Chemistry. Blakiston, Philadelphia, 1907.

Reduction indicated by +.

No reduction during ten minutes in boiling water bath indicated by −.

levulose, lactose, and maltose solutions decolorized methylene blue, however, in one or two minutes in N/1000 NaOH, but raffinose, inulin and dextrin solution required N/100 NaOH while sucrose, mannitol, and dulcitol, failed to decolorize methylene blue in even N/3 NaOH.

These results with ten representative carbohydrates of reputable purity suggested the following attempt to correlate methylene blue reduction with that of copper sulphate in Fehling's test as in table 1.

There is apparently a well defined correspondence between those carbohydrates whose reducing action is shown in Fehling's

test and those which reduce methylene blue in N/1000 NaOH. These carbohydrates are also most susceptible to alkaline-hydrolysis. The trisaccharid raffinose and the poly-saccharids inulin and dextrin are generally considered not to give Fehling's test; they are less easily hydrolysed by alkalis, and they require therefore a stronger concentration of alkali to reduce methylene blue. The disaccharide, sucrose, and the alcohols, dulcitol and mannitol, are especially resistant to alkalis: they respond therefore to neither Fehling's nor the methylene blue test. But preliminary treatment of sucrose with N/100 HCl readily hydrolyses it and the overneutralization of such a mixture to N/100 alkalinity causes it to decolorize methylene blue quickly on heating.

Quantitative relations

In many of the experiments up to this point the importance of quantitative relations was recognized.

We have just seen that a minute quantity of alkali (N/1000 NaOH) suffices to insure decolorization of certain carbohydrates in 1 per cent solution with 0.00005 gram methylene blue per cubic centimeter. The rapidity of decolorization of glucose solution varies according to the concentration of alkali, which, if sufficiently strong, effects the destruction of color without heating: furthermore, less alkali is required to effect the loss of color under anaerobic conditions than in the presence of the air. The following is abstracted from a protocol covering an experiment with 1 per cent glucose, 1:10,000 methylene blue, of varying degrees of alkalinity as indicated, placed in constricted tubes with marble seals and read after twenty-four hours incubation at 37°C. without preliminary heating.

TUBE NUMBER	NaOH	ABOVE MARBLE	BELOW MARBLE
1	N/60	Nearly colorless	Slightly yellow
2	N/120	Blue	Colorless
3	N/240	Blue	Nearly colorless
4	N/480	Blue	Blue

On boiling five minutes all were decolorized above and below except tube 4. This experiment thus illustrates not only the point just mentioned but also our frequent observation that very weakly alkaline solutions are likely to fail to decolorize if allowed to stand exposed to the air long before use, probably owing to neutralization by CO_2. This is a point to which we shall return.

As to variations in dye content with N/1000 NaOH and 1 per cent glucose, 1:1000 and 1:10,000 methylene blue failed to decolorize in this particular experiment while solutions containing 1:100,000 did so. The weaker the concentration of dye, the less alkali is required.

With N/1000 NaOH and 1:10,000 methylene blue variations in glucose from 0.15 to 20 per cent appeared to make little or no difference in decolorization, yet further dilution and variations in alkalinity and dye content showed distinct effects, to which reference will now be made for it is apparent that the three reagents necessary in a test for the decolorization of methylene blue by heating bear a definite quantitative relation, one to another. Briefly, the amount of alkali required bears an inverse relation to that of glucose but the necessary amounts of these two reagents bear a direct relation to that of methylene blue. The more alkali the less glucose is required and vice versa, but the more methylene blue the more glucose or alkali is required. Those relations are best displayed in the following experiment:

For the purpose of this and several similar experiments a copper water bath with a support providing for 10 rows of 10 perforations each to hold test tubes was used. The tubes were of uniform size as to length and bore; they were carefully cleaned and placed in the support in rows corresponding to the record marks of table 2, one tube for each mark. To each were first added 7 cc. neutral distilled water and 1 cc. of an aqueous methylene blue solution 10 times the strength required in that particular section of the experiment. Solutions 10 times the strength of glucose required in each of the vertical rows and of sodium hydroxide in each of the horizontal rows were prepared and of these 1 cc. each was added to each tube in the test. In such

an experiment it is always important to add the alkali last to avoid any considerable action of a concentration greater than that indicated by the recorded data. The total volume of liquid in each tube was 10 cc.

The support with the tubes was then placed in the bath filled with boiling water and the boiling continued for ten minutes, when the support with the tubes was removed and the color or lack of color in the solutions recorded. The reading was repeated five and fifteen minutes after removal from the bath.

To facilitate the manipulation and observation of so many tubes when an important time element is involved it was necessary to divide the experiment in point of time into three sections corresponding to the different quantities of methylene blue used; conditions were duplicated as far as possible in each section with the exception of the quantity of dye, even to the use of dilutions from identical solutions of the three reagents. Also while the data submitted were secured during the space of one afternoon, the tests were repeated several times on other occasions with essentially similar results.

The lines drawn in table 2 indicate the division at each reading between those tubes showing definite color and those not showing color. Next the line on the colored side there were always tubes partially decolorized. As the tests were exposed to room temperature and the air the division line had to be moved in the direction of stronger alkali and stronger glucose, in short, those solutions containing least sugar and least alkali were last to decolorize and first to regain their color.

Table 2 shows also that larger quantities of glucose and alkali are required for the decolorization of a larger quantity of methylene blue and, further, that a decrease in alkali is compensated for by an increase in glucose. Roughly, within certain limits a five-fold increase in glucose permits halving the alkali and vice versa. It is not difficult to understand the direct correspondence between the amount of dye decolorized and the amounts of glucose and alkali required upon the theory that a definite quantity of some substance or substances produced by the action of alkali on glucose and other susceptible carbohydrates is necessary

as a matter of chemical equivalence but an attempt to apply the Guldberg-Waage mass law was not successful.

The present status of our knowledge of the changes which monosaccharids undergo in the presence of alkalis, so well sum-

TABLE 2

Decolorization and recoloration of varying concentrations of methylene blue in relation to varying concentrations of glucose and alkali

NaOH	METHYLENE BLUE 1:1000						REMARKS
N/100	−	−	+	+	+	+	After 5 minutes
N/200	−	−	−	+	+	+	At once
N/400	−	−	−	−	−	−	
N/800	−	−	−	−	−	−	
N/1600	−	−	−	−	−	−	
N/3200	−	−	−	−	−	−	
	0.004	0.02	0.1	0.5	1.0	2.0	Per cent glucose
	METHYLENE BLUE 1:10,000						
N/100	−	−	+	+	+	+	After 15 minutes
N/200	−	−	+	+	+	+	After 5 minutes
N/400	−	−	−	+	+	+	At once
N/800	−	−	−	−	+	−	
N/1600	−	−	−	−	−	−	
N/3200	−	−	−	−	−	−	
	0.004	0.02	0.1	0.5	1.0	2.0	Per cent glucose
	METHYLENE BLUE 1:100,000						
N/100	−	+	+	+	+	+	
N/200	−	+	+	+	+	+	After 15 minutes
N/400	−	+	+	+	+	+	
N/800	−	−	+	+	+	+	After 5 minutes
N/1600	−	−	−	+	+	+	
N/3200	−	−	−	−	+	+	At once
	0.004	0.02	0.1	0.5	1.0	2.0	Per cent glucose

− indicates no reduction—a blue solution.
+ indicates reduction—a colorless solution.

marized by Woodyatt (1915, 1918), indicates a tremendous variety of reactions according to the sugars concerned, the concentration of hydroxyl ions, degree and time of heating, presence

and absence of air, etc. In general, the basis laid by Lobry de Bruyn (1895), Lobry de Bruyn and Van Ekenstein (1895, 1896, 1897) Nef (1907), Mathews (1909), Henderson (1911), Glattfeld (1913), and others, indicates two groups of products resulting from alkali treatment, first, isomers as a result of the action of weak concentrations, low temperature, etc., and second, split products as a result of stronger concentration and higher temperature. Weak alkalis are transformative, strong alkalis destructive. The literature indicates clearly that glucose ionizes in the presence of alkali as a weak acid, which can be readily shown by colorimetric determination of the change in H^+ ion concentration of alkaline buffer solutions to which glucose is added. Alkali upsets the stability of the molecule causing the formation not only of all the possible isomers, but of metallic glucosates, and sugars of one, two, three, four, and five carbon atoms as well as oxy-acids.

The fact that decolorization occurs in the presence of minute quantities of alkalis might seem to speak strongly for some isomer as responsible for decolorization. But since isomers as well as the original sugar are destroyed by higher concentrations of alkali and these decolorize more readily than low concentrations we cannot entertain this idea seriously. Also the decolorization of methylene blue in similar concentrations of glucose and levulose depends upon identical concentrations of alkali; thus 0.1 per cent solutions of these sugars were decolorized in N/800 NaOH but not in N/1600 NaOH during ten minutes boiling.

Similarly the temptation to explain the possible reduction of alkalinity in the test almost to the vanishing point, by increasing the glucose content, as a result of the adulteration of glucose with effective isomers or split products is checked by the observation that even 20 per cent solutions of glucose without alkali fail to decolorize methylene blue on prolonged boiling.

On the other hand we are unable to exclude split products as the effective agency when alkali is present. Methylene blue solutions caramelized by boiling a few minutes in N/10 or stronger NaOH, and, when neutralized or even slightly acidified, and allowed to regain their color (yellow + blue = green), can be

decolorized (yellow) in this condition by further boiling; furthermore, *prolonged* boiling of glucose, levulose, and lactose in strongly acid solutions also results ultimately in more or less complete decolorization of methylene blue.

Some of the organic acids were noted above as failing to furnish conditions necessary for the decolorization of heated methylene blue solutions even in the presence of glucose. Alone in N/10 concentration, neutralized with equivalent amounts of N/10 NaOH, and alkalinized to N/10 NaOH, they also fail. Neither formaldehyde, a building stone of glucose, nor ethyl alcohol, one of the most frequent fermentation products of glucolysis, in 5 per cent solution, acidified with HCl to N/10, neutral, or alkalinized to N/10 NaOH, causes the decolorization of methylene blue solutions containing 1 part per 100,000 on boiling. Other products of alkali glucolysis must be tested if we are to fasten the responsibility for the decolorization of methylene blue upon a definite single substance. Our present speculations lead us to suspect that decolorization of methylene blue depends upon those conditions which liberate nascent hydrogen and, that the formation of metallic glucosates by alkalis is somewhat analogous in this respect to the action of HCl on zinc. Or, it may be that the hydrogen required for the reduction of methylene blue to its leuco-base is derived from the dissociation of water and corresponds to the equivalent oxygen uniting with the residue of the sugar molecule, according to Nef's theory.

Two per cent Witte's peptone solutions and 2 per cent agar solutions with 1:100,000 methylene blue are decolorized by heating with alkali. But with peptone, at least 1 part N/1 NaOH in 128 had to be present, owing possibly to the considerable buffer action of peptone. With agar solutions (pH = 7) decolorization occured with 1 part N/1 NaOH per 200 agar but not with 1 part per 250, although agar is supposed to have little or no buffer action according to Clark and Lubs (1917). Addition of 0.5 per cent glucose did not permit decolorization in less alkali than in controls without glucose, in fact the presence of agar inhibits decolorization in concentrations of alkaline glucose solution which will readily decolorize without the agar.

In the decolorization of methylene blue temperature is a factor; heat plays a double rôle, driving out oxygen by lowering the solubility point and accelerating the chemical reaction between alkali and organic matter.

Sunlight also effects the decolorization of methylene blue but this factor is mentioned here only as a disturbing influence which has been avoided in the experimental work. Lasareff (1912) and Gebhard (1912) have shown that the bleaching effect of light is most intense in the absence of oxygen; the color returns in the dark in the presence of oxygen providing exposuce was to wave lengths less than 620 $\mu\mu$ but otherwise does not.

Table 3 displays the results of an experiment showing that the return of color to decolorized methylene blue agar in bright sunlight is considerably less rapid than in diffuse light or in the dark.

TABLE 3

Depth of colored band at top of 2 per cent agar with N/100 NaOH *at different time intervals after decolorization, in varying light intensities*

	AFTER FOLLOWING NUMBER OF MINUTES REMOVAL FROM BATH:							
	15	30	45	60	120	180	240	300
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Sunlight	0.5	0.6	1.0	1.5	2.6	3.4	4.1	4.9
Diffuse-light	0.5	1.1	2.0	2.5	3.8	5.0	5.5	6.0
Dark	0.8	1.5	2.1	3.0	4.1	5.2	5.6	6.2

As to the decolorization of methylene blue by living cells this discussion does not particularly concern itself further than to note with Jordan that "anaerobes will grow in media where . . . . reduced methylene blue shows no trace of reoxidation." They will grow also in undecolorized methylene blue but observations of many tests have shown no instance where such growth was unaccompanied by decolorization. While, as Ricketts (1904) has mentioned, we cannot regard the reduction of methylene blue as a definite test for living cells, as Ehrlich and others have suggested, since methylene blue becomes leucomethylene blue when its affinities for hydrogen have been satisfied, whether through reduction by living or non living matter,

yet in the known absence of non living reducing agents, the decolorization of methylene blue in culture media may be taken as a fair indication of anaerobic growth where the conditions of anaerobiosis are such as not in themselves to decolorize the dye. The failure of certain streptococci to decolorize methylene blue in milk as sherman and Albus (1918) found, appears to be a matter of inhibition; it is interesting to note Brown's (1920) observation that some of these forms will develop in the depths of agar containing decolorized methylene blue but not in the colored band near the surface; contrary-wise it is possible for many organisms to grow aerobically upon media colored with methylene blue without decolorization. The possible rôle of adsorption of methylene blue by bacterial bodies in its relation to true reducing processes still remains to be investigated.

Recoloration of methylene blue

Whereas we are able only to speculate as to the basic explanation of these various phenomena a knowledge of them enables us to guage correctly the concentration of ingredients in the use of methylene blue as a criterion of anaerobiosis. Such use depends, as already noted, upon the recoloration of decolorized methylene blue in the presence of air, and the failure of recoloration when air is excluded. But recoloration does not occur in glucose solutions in alkali stronger than N/32 in which marked caramelization has occurred, nor in peptone more strongly alkaline than N/16, nor in agar sufficiently alkalinized to prevent solidification; neutralization of such glucose solutions permits recoloration, however (Yellow + blue = green).

As a general rule, the delicacy of methylene blue as a criterion of anaerobiosis varies directly as the kind and amount of reducing agent employed, and the temperature used to effect decolorization, and inversely as the alkalinity of the solution. As shown in table 2 those decolorized solutions last to lose their color were first to regain it. In general a moderate concentration of glucose, e.g., 0.5 to 2 per cent with a low concentration of alkali (N/500 to N/1000 NaOH) gives the best results for tests involving

liquids; 2 per cent neutral agar, plus 1 part N:1 NaOH per 100 is satisfactory for tests involving solid media.

There is a possible fallacy in the use of too weakly alkaline solutions, namely, that on standing they cannot be decolorized by boiling. By exposing all the seven possible combinations of one, two, or all, of the three factors, glucose, alkali, and dye, for forty-eight hours, and then adding those lacking in each of six of these, it can be shown readily that only those originally containing alkali deteriorate; that is, deterioration consists in loss of alkalinity. The test solution must be freshly alkalinized, though the glucose methylene blue or agar methylene blue may be kept as stock solutions. Loss of ability to decolorize might conceivably be attributable to acid in the glassware though I have never encountered this factor knowingly. The change of reaction is most reasonably attributed to absorbtion of atmospheric carbon dioxide. An easy proof of change in reaction of faintly alkaline solutions on exposure to air is afforded if one heats N/1000 NaOH colored with phenolphthalein in a constricted tube with marble seal in a bath of boiling water; this dye is not affected by such heating. But on cooling for several hours the color above the marble fades while that below remains. Or, drawing air through such a colored solution causes it to fade, through change of reaction, but if the air be washed by bubbling through several tubes of strong lye to remove CO_2, with the efficacy of such removal tested by passage through clear lime water, the phenolphthalein test solution remains alkaline. A repetition of this last experiment with a decolorized methylene blue solution gives the same result, i.e., recoloration, with air containing CO_2 and air freed therefrom, except that the solution recolorized with the latter continues susceptible to repeated decolorization longer than with the former. This proves that CO_2 is not the only factor in recolorization of methylene blue as it is in the change of reaction in the phenolphthalein experiment. If the air be carefully washed in several successive mixtures of alkaline pyrogallol so as to remove both carbon dioxide and oxygen its passage through a decolorized methylene blue solution does not cause the return of color. In short, there

are two possible factors in the recoloration of methylene blue by exposure to air, oxygen and carbon dioxide—two processes, oxidation and acidification.

Since the reaction rests unquestionably upon a quantitative basis, even though we know nothing of the absolute values in oxygen and carbon dioxide concerned, the volumes of test solution in relation to surface exposure, where time marks the progress of recoloration, is of great importance in comparative tests. With equal surface exposure large volumes regain their color more slowly than small volumes. In all cases care has been taken to use the same size tubes and identical volumes in a given experiment unless otherwise stated. Differences in volume between experiments account readily for certain apparent discrepancies in actual observations of time required for recoloration.

Application to methods of culture

With these data at hand tests have been made of a great many methods of cultivation, in which connections I gladly acknowledge the aid of my student, Miss Margaret Eakin. Here, as in the culture of anaerobic microorganisms, we have to distinguish between the factors of oxygen tension reduction and of reduced oxygen tension maintenance.

We have referred already to the literature on biological reduction of methylene blue; to this we may add that our experiments show the general possession by living cells of the property of reduction. This property is common to many aerobes and anaerobes, so in symbiotic mixtures, methylene blue is reduced as a matter of course. With a broth culture of hay bacillus in an external rubber stoppered tube and a smaller internal tube containing slanted 2 per cent agar with 1:100,000 methylene blue and N/100 NaOH analogous to the method of Salomonson (1889), only partial reduction was obtained in twenty-four hours at 37°C. and similar tubes of nutrient agar inoculated with *B. botulinus*, *B. tetani*, *B. welchii* and other obligative anaerobes failed to yield satisfactory surface growth. Failure of complete decolorization here is correlated with refusal of strict anaerobes

to multiply. This method is well known to be adapted to the culture of microphilic aerobes, however.

Plant and animal tissues also reduce methylene blue in the depths of liquid media. In one instance a piece of sterile guinea pig kidney under mineral oil kept methylene blue decolorized in its immediate neighborhood at 37°C. for 196 hours whereas the control without tissue but with an equivalent depth of oil was completely recolored in thirty minutes.

Many investigators, as already noted, have referred to the decolorization of methylene blue by animal and plant tissues as well as by various inert substances in culture media as indicating anaerobic conditions therein. Of these, Zinsser, Hopkins and Gilbert (1915) recognized most clearly that we have to deal here with another process in addition to reduction, namely adsorption. They were unable, by extraction of animal organs, to secure any reducing substance whatever apart from the tissues and concluded that adsorption is mainly responsible for the loss of color in media containing methylene blue in the presence of such agents. This conclusion was strengthened by their observation that heated tissues are nearly, if not quite, equal to unheated tissues for this purpose. Similar observations were previously made by Wrzosek (1907), Liefmann (1907), Guillemot and Szczawinska (1908), and Hata (1908) but it is doubtful if any of these workers appreciated the important rôle of adsorption.

It is possible, as I shall show presently, to extract reducing substances from both plant and animal tissues, and in this important respect their action upon methylene blue differs from that of inert particulate substances such as sand.

When a small piece of potato was placed in an aqueous solution of methylene blue (1:100,000) at room temperature, the solution adjacent to the tissue lost its color within two to three hours and within a few more hours the test tube showed a lightly colored bluish liquid in which the potato fragment was slightly tinged with blue, most prominently at its uppermost end. No recoloration occured in such a partially decolorized solution on exposure in a Petri dish nor could it be decolorized by boiling except on alkalinization. In contrast, the potato fragment became mark-

edly bluer on exposure, and if cut into displayed a decreasing intensity of dye in the interior, the color deepening rapidly in contact with the air. Such an experiment may be interpreted as indicating adsorption plus reduction, the latter occurring mainly, if not exclusively, within the plant tissue.

Acid (N/100 HCl) and alkaline (N/100 NaOH) solutions gave similar results. Heating such a series immediately in the boiling water bath resulted in decolorization of the alkaline solution only. Exposed to the air in the tube the color returned to this solution on cooling in an intensity practically equal to that of the neutral and acid solutions. Adsorption proceeded in all three and did not seem to be notably accelerated by the heating. Previous boiling of the potato fragment seemed to have no influence on the result.

Extraction by boiling a 1 gram fragment of potato in 1.0 cc. of N/10 HCl, neutral water, or N/10 NaOH for ten minutes and decanting the supernatant fluid yielded a solution containing a reducing substance for methylene blue which could be demonstrated by its decolorization on boiling in N/20 alkaline solution. The color readily returned to such solutions on exposure to the air in a Petri dish. The method of extraction suggested that the substance extracted was probably starch which assumption was substantiated by the iodine test. Starch reduces methylene blue on boiling in alkaline solutions.

Experiments with animal tissues, such as rabbit and guinea pig liver, in aqueous solutions of methylene blue gave results apparently identical with those recorded for plant, i.e., potato tissues. The solutions, acid, neutral and alkaline, became decolorized in the immediate neighborhood of the tissues within a few hours and almost completely, throughout, in twenty-four hours. The nearly colorless solutions separated from their tissues did not regain their color on exposure to the air nor could they be completely decolorized by heating except in the case of the alkaline solution. Alkalinization of the neutral and acid solutions, however, facilitated their rapid decolorization by heat. The tissues became slightly tinged with blue during contact with the dye solution and quickly colored on exposure to the air,

both on the surface and in the exposed depths. These phenomena coincide exactly with those observed for the plant tissue and point to the same two processes, adsorption and reduction.

But when it came to extraction of the reducing substance from the animal tissues it was found that the solutions from freshly boiled liver, whether acid (N/10 HCl), neutral, or alkaline (N/10 NaOH), failed to decolorize methylene blue added to them, even when strongly alkalinized and heated further. Immediate decolorization upon the addition of a trace of glucose proved the suitability of the general conditions of the test for the proof of a reducing agent. But kept in the ice chest overnight either with or without previous boiling, and in acid, neutral or alkaline solutions and then further boiled immediately previous to separation of the clear supernantant fluids, guinea-pig liver yielded a reducing substance to the fluid capable of decolorizing methylene blue under the influence of heat in alkaline solutions. Guinea pig kidney also gave a similar result in neutral distilled water; acid and alkaline extractions of kidney were not tried.

The results with these animal tissues differ from those with potato, both in respect to the relative ease of extraction of the reducing agent in the latter case, and probably in regard to its chemical nature. There is little reason to doubt that the reducing substance extracted from potato is starch; the chemical nature of that from the animal tissues is only conjectural. We may say definitely that it belongs to the non-heat-coagulable extractives, that it is not materially affected by relatively strong acids and alkalis, and that it escapes from the tissue into the solution during sixteen hours maceration in the ice chest or at room temperature, with or without previous boiling. Furthermore, and this may be the point overlooked by Zinsser, Hopkins and Gilbert (1915), a necessary condition for decolorization of methylene blue by heat in the presence of either the reducing substance from potato or that from rabbit and guinea pig liver and kidney is an alkaline reaction. Solutions so decolorized regain their color on exposure to air.

Thus in considering the action of such plant and animal tissues in anaerobic culture media from the standpoint of their effect

on methylene blue we have to recognize that both adsorption and reduction are concerned.

To complete a representative study of porous substances used in the cultivation of anaerobic organisms I have selected white sea sand. There has been a strong suggestion in such recent work as that of Douglas, Fleming and Colebrook (1917) that the principal value in plant and animal tissues added to culture media for the cultivation of obligative anaerobes lies in their provision of interstices which by their minute size serve to prevent diffusion of oxygen as well as to afford secluded foci for the initiation of growth, and this view has much to commend it. They have shown, indeed, and others as well as ourselves have confirmed, the value of various inert insoluble substances added to media in place of animal and plant tissue.

When I attempted the treatment of simple methylene blue solutions with sand, results startlingly like those with tissues were secured except that there was no reduction in the depths of the sand. In brief, adsorption is the sole process concerned here, and it occurs aerobically as well as anaerobically. In a Smith fermentation tube filled with an aqueous methylene blue solution and shaken up with sand which settled into the neck, marked decolorization occurred in both the open and the closed arms.

Of course it was impossible to "extract" a reducing agent from sand. But so far as the solution itself was concerned it behaved exactly like that treated with tissue; with this exception, that some reducing agent such as glucose, as well as an alkaline reaction had to be provided in order to secure complete decolorization by heating.

Whereas sand of itself has no true reducing action, there is no doubt of its efficacy as a means of maintaining reduced oxygen pressure, as we may judge from the persistence for many days of decolorization in the closed arm of a fermentation tube provided with a slightly alkaline glucose solution of methylene blue and a sand seal in the neck of the tube. The sand seal with suitable culture media in the fermentation tube is also quite satisfactory from the cultural standpoint.

In summary, the difference between plant and animal tissues and inert substances such as sand are the differences between more or less soluble organic substances and insoluble inorganic substances. No doubt, plant and animal tissues may serve the same mechanical purposes as sand; in addition they may supply nutrients to the medium, buffer substances and possibly even "vitamins." With plant and animal tissues in media, otherwise lacking in reducing substances, these may be of supplementary importance in the cultivation of obligate anaerobes. Finally, unless we are willing to concede some importance to adsorbtion as a factor in anaerobiosis, sand and other inert porous substances may serve only as a means of maintenance of reduced oxygen tension, i.e., as seals, whereas tissues may serve not only this purpose but may actually aid in the reduction of oxygen tension in addition to the nutritive functions they fulfill. We may emphasize the importance of heat in this connection since the existence of a true self active reducing agent as distinguished from the phenomenon of adsorbtion seems as yet unproved.

As to physical reduction, i.e., ebullition, the data already presented bear testimony to the efficacy of boiling. In these tests, as in the actual culture of anaerobes, boiling is often an essential preliminary procedure in the test. The use of both liquid and solid deep media so decolorized shows the first return of color at the top and proves the importance in the case of liquid solutions, of such factors as narrowness of bore in the tube volume of solution, the effect of diffusion currents, etc. With solid media these are not so important.

In 1, 2 and 3 per cent agar, with N/100 NaOH and 1:100,000 methylene blue, decolorized by boiling in standard culture tubes of 1.5 cm. bore, the depth of the blue band at the top of the agar at various intervals appeared as in table 4.

Apparently variation of agar content, within the limits of 1 to 3 per cent makes only a little difference in the rate or depth of recoloration. We may point out that the depth of blue color at the top of the agar column corresponds roughly to that in a deep glucose agar stab or shake culture which is free from growth though there are doubtless variations according to species, and

perhaps nutrient conditions, as Burke (1919) has mentioned. On standing longer the blue band thickens, and it is suggested that the distance from the surface at which anaerobic growth commences is determined partly by the rapidity of multiplication permitted by the nutritional conditions of the culture.

Deep tubes of agar, in which recoloration of decolorized methylene blue is occurring, present the phenomenon of rhythmic banding, i.e., Liesegang's rings. This subject has been studied recently by Holmes (1918) in other cases, but no one, so far as I am aware, has investigated the phenomenon in the case of methylene blue, for which no really satisfactory explanation is available.

TABLE 4

Depth of returning blue band in decolorized methylene blue agar of varying densities

AGAR	MINUTES					
	5	10	20	30	40	50
per cent	mm.	mm.	mm.	mm.	mm.	mm.
1	0.4	0.5	0.6	1.8	2.6	3.3
2	0.4	0.5	0.7	1.8	2.5	2.8
3	0.6	0.8	0.8	2.5	3.5	4.5

AGAR	HOURS				
	1	2	4	10	21
per cent	mm.	mm.	mm.	mm.	mm.
1	3.6	5.5	7.5	11.2	16.5
2	3.2	5.3	7.2	11.0	16.0
3	5.0	7.5	10.0	14.0	19.8

Other methods of deep culture involving solid or semi-solid media present phenomena analogous to those observed with deep agar tubes. Thus gelatin and deep brain media with methylene blue remain decolorized in the depths for several days after heating but the immediate coloration in the uppermost layer gradually extends downward as oxygen and carbon dioxide are absorbed. Corresponding to the usual failure of cultural tests with unprotected liquid media, alkaline glucose solutions of methylene blue regain their color on exposure to

air soon after boiling. But large flasks of solution or very slender deep tubes of such decolorized solutions remain decolorized for some hours—sufficiently long, indeed, for anaerobic growth to be initiated in suitable media heavily inoculated under similar circumstances. In the Smith fermentation tube efficacy for anaerobic culture, or maintenance of decolorization of methylene blue, depends largely upon the bore at the bend and satisfactory results for either cannot be secured without the use of a special seal, such as sand, tissues, etc. In some unprotected tubes the color returned to the solution in the closed arm in fifteen minutes, in others in sixty minutes and in still others after several hours; with sand seals the dye color returned only in the open arm and then gradually faded even here through adsorption.

The method of deep colony culture between the nested halves of a Petri dish when tested with methylene blue showed a progressive recoloration of the decolorized dye from the periphery inward, except when protected by a paraffine or vaseline seal.

There is no phase of the methylene blue problem to which we have given more careful attention than its use in connection with insoluble liquid seals, i.e., hydrocarbon oil, wax and grease. The widespread use of these substances as a means of excluding oxygen together with the theoretical and practical objections to their use, especially in the case of oil, have justified a searching examination of this matter. The results with methylene blue only serve to support the conclusion that liquid hydrocarbons are to a degree superfluous and inefficacious means of maintaining anaerobiosis; on the other hand the waxes and semisolid grease seals are more satisfactory for certain purposes, from the standpoint of air exclusion, though inferior to mechanical seals in the matter of convenience and cleanliness.

In the first place an alkaline glucose solution of methylene blue does not decolorize at 37°C. under 2.5 cm. depth of mineral oil, although it may be mentioned here that it does so readily in a few hours under the marble seal in a constricted tube, or under a cover slip in a plain tube, or under a thin layer of paraffine.

I have shown repeatedly with different samples of mineral

oil that the color returns to alkaline glucose methylene blue solution decolorized under varying depths of oil by heating in a boiling water bath almost as soon as without the oil. The following instance illustrates this point. Two per cent glucose (Pfanstiehl) with N/500 NaOH and 1:100,000 methylene blue (Grübler) in aqueous solution was placed in equal depth (3 cm.) in similar culture tubes of $\frac{1}{2}$ inch diameter and covered to the depths noted with "Fulmor" oil, a white neutral mineral oil prepared by the Fuller Morrison Company of Chicago; a constricted tube with the same dye solution and marble seal was included for a control. It should be noted that the diameter of this tube was about twice, and the surface exposure of liquid therefore 4 times, that of the other, thus offering even greater opportunities for rapid recoloration, which was observed above the seal. All were decolorized throughout by heating two minutes in the boiling water bath and readings made as follows on the removal therefrom.

DEPTH OF OIL	ONE-QUARTER HOUR	ONE HOUR	TWO HOURS	SIXTEEN HOURS—(BOILED TEN MINUTES)
cm.				
5	Colorless	Slightly blue	Blue	Nearly colorless
3	Colorless	Slightly blue	Blue	Nearly colorless
1	Colorless	Slightly blue	Blue	Slightly blue
0	Slightly blue	Blue	Blue	Blue
Constricted tube	Above—Blue	Blue	Blue	*
	Below—Colorless	Colorless	Colorless	*

* Not boiled with other tubes—still colorless below after six days.

This representative experiment indicates that oil is much less efficacious than sometimes assumed as a means of oxygen exclusion. The progressive ease of repeated decolorization in relation to depth of oil suggests that carbon dioxide is excluded somewhat better but one must not lose sight of the fact that the maximum depth of oil in this experiment is much greater than ordinarily used.

I have noted elsewhere (1915) that, culturally, the growth of obligative anaerobes is delayed under oil except where relatively large inocula are used. In certain experiments, comparing the efficacy of the marble and oil (2.5 cm.) seals in constricted tubes with identical media, growth has been negative with the oil seal in twenty-four to forty-eight hours at a million times the dosage showing definite growth under the marble. Continued observation of the oil tubes has usually decreased the disproportion, however. Although these experiments suggest an inhibitive action of the oil this was not substantiated by comparing progressively diluted cultures under both the oil and marble with a similar set under the marble only; in this case equivalent dilutions developed in parallel order.

In this connection it was interesting to study the effect of filling a constricted tube with alkaline methylene blue solution and oil in such a way that the marble seal lay in the oil. The results of a carefully controlled experiment are summarized herewith, the solutions having been decolorized first in the usual way by heating and removed for observation.

	Ten minutes
Tube 1—No seal	Solution blue
Tube 2—Marble in solution	Blue above Colorless below—remained so for over two weeks.
Tube 3—Marble in oil	Solution blue
Tube 4—Oil alone	Solution blue

Tube 3 in addition to showing this remarkable result also shows regularly, in such an experiment, a striking and fairly permanent emulsification of water in oil which has been made the subject of a special monograph by the writer (1917).

But it was most disconcerting to find that the marble placed in the oil fails to protect the decolorized solution from recoloration on cooling and suggests that the effect of heating a solution in contact with oil is to drive the oxygen from the solution in which it is less soluble, into and possibly to some extent through the oil, in which it is more soluble, and that on cooling there is a return of some of the oxygen from the oil to the solution.

A duplication of this experiment using phenolphthalein instead of methylene blue showed that CO_2 also is probably similarly concerned; for with the marble seal in the solution the alkalinity of that portion below the seal was protected for over twenty-one hours as against a failure above the marble but below the oil within 1 hour, while with the marble in the oil the solution was only faintly alkaline at one hour and frankly acid at three and one-half hours.

It is impossible to attribute any change in reaction directly to contact with the oil in view of an experiment with oil layered on standard buffer solutions of known acidity (pH = 5, 6, 7, 8, and 9) and colored with brom-cresol purple and cresol-red in their respective ranges for comparison with identical solutions with out oil; there was not the slightest evidence of changed reaction either after shaking together cold, or during, or immediately after heating.

An attempt to make a better showing for the oil by heating the mixture of alkaline glucose methylene blue solution in the autoclave for twenty minutes at 25 pounds pressure (267°C.) gave no better results. Neither was separate heating of solution and oil either in the boiling water bath or in the autoclave, followed by immediate layering, as efficacious in preventing the return of color as heating together in the water bath.

Vigorous boiling of the solution under the oil by the cautious use of a 110°C. saturated salt solution while accelerating the rate of decolorization failed to show any material advantage in excluding the air as judged by the time in which the color returned.

Several attempts to layer oil at or near its own boiling point (about 300°C.) upon decolorized solution at 100°C. resulted disastrously in breakage of glassware and almost explosive scattering of hot oil. Cooled rapidly to about 120°C. in a few exeriments I had the gratification of seeing the solution under the oil, even without the marble seal, remain completely decolorized for nearly twenty-four hours; with the marble seal in the oil it remained so for much longer. In still other experiments where the oil was heated to boiling and allowed to cool even for a few minutes, to as low as 60°C. and then immediately brought

up to 100°C. and layered upon the hot solution the color returned to the latter in about half an hour as in the case of oil heated with the solution in a boiling water bath.

If one increases the depth of solution in a tube without oil, several times over that of a similar tube with oil, both being equally decolorized by boiling, the former may be seen to regain its color even before the latter.

These experiments lend little support to the use of oil as a means of anaerobiosis and limit the technic where it is used to layering on of freshly boiled oil quickly cooled nearly to 100°C., but even in this case it is less efficacious than the marble seal.

In none of the experiments with alkaline glucose methylene blue solution has there been any evidence of absorption of the dye by the oil. Methylene blue is insoluble in oil. A bluish tinge sometimes observed in the oil layer is really due to the dye dissolved in a film of water which separates the oil from the glass wall as I have mentioned elsewhere (1917) or, in the case of oil-dye solutions actively boiled over the free flame or in a strong salt solution bath, to emulsified water holding the dye in suspension. A suggestion that the dye might be absorbed in the form of the colorless leuco-base was proven erroneous by pipetting off the oil from the tube of decolorized dye solution into a tube of distilled water; on exposure to air the color returned at once to the original dye solution whereas the water and the oil overlying it remained quite colorless.

Experiments analogous to some of those with the liquid solution have been performed with 2 per cent neutral agar made alkaline by the addition of 1 cc. N/1 NaOH per 100 and colored with 1 part methylene blue per 100,000 as offering a roughly quantitative measure of the rate of air absorption which is indicated by the thickness of the blue band that appears at the top and deepens as exposure continues. Another advantage of this means of test is that disproportionate volumes do not introduce time differences into the observations of recoloration as they do with a liquid test solution, yet in both cases the volumes and areas exposed in different tubes have been kept identical for comparative purposes except where otherwise noted.

The importance in such observations of having a sufficient quantity of test solution is shown in the following experiment which offers a comparison of the results with equal ratios but differing absolute quantities of test solution and seal.

The liquid test solution was the usual 2 per cent glucose, with N/500 NaOH, and 1:100,000 methylene blue; the agar test solution was 2 per cent agar with N/1 NaOH and 1:100,000 methylene blue; the seal was vaseline. The mixtures were made in tubes of 10 cm. diameter and heated in a boiling water bath for a few minutes to decolorize. They were allowed to stand overnight at room temperature and then examined for recoloration. The results appear in table 5.

TABLE 5

Relation between absolute and proportional quantities of test solution and seal

VASELINE	SOLUTION	RESULT	VASELINE	SOLUTION	RESULT
Liquid test solution					
cc.	*cc.*		*cc.*	*cc.*	
1	10	Colorless	1	10	Colorless
1	5	Colorless	2	10	Colorless
1	2	Blue	5	10	Colorless
1	1	Blue	10	10	Colorless
Agar test solution					
1	10	Blue, 1 mm.	1	10	Blue, 1 mm.
1	5	Blue, 1 mm.	2	10	Blue, 1 mm.
1	2	Blue, 1 mm.	5	10	Blue, 1 mm.
1	1	Blue	10	10	Blue, 1 mm.

The logical explanation of this result appears to lie in the assumption that sufficient oxygen or carbon dioxide is retained in the seal to recolor a small amount of test fluid previous to setting of the seal but not sufficient to recolor a larger amount.

Boiling an agar solution under 3 cm. of oil, in a water bath, heating to 25 pounds steam pressure (267°C.) in the autoclave for twenty minutes, or layering the separately heated oil and agar solutions, made only a slight difference in the rate of return of color at the oil-agar surface, which is almost as rapid as without the oil.

Neither heating the oil in the boiling water bath nor boiling (about 300°C.) over the free flame appeared to affect the density of color in the superficial colored layers of alkaline, 2 per cent agar containing methylene blue 1:100,000. This experiment negatives the suggestion that de-aerated oil is able to abstract oxygen and carbon dioxide from media containing them. Also, whereas melted alkaline agar colored with methylene blue and poured without further heating to decolorize into tubes, loses its color to within 1 to 2 cm. of the surface in a few hours, the immediate layering of such a solution with mineral oil makes only a barely measurable difference in the thickness of the band of color at the top.

TABLE 6

A comparison of mineral oil and paraffine as seals against the recoloration of alkaline methylene blue agar

	HOURS						
	0.25	2	6	24	45	70	96
	cm.	cm.	cm.	cm.	cm.	cm.	cm.
No seal	0.5	0.7	0.8	1.5	1.7	2.0	2.5
Mineral-oil	0.3	0.5	0.6	1.0	1.5	1.9	2.5
Paraffine	0.2	0.3	0.4	0.6	1.1	1.1	0.9

Depth of agar, 7 cm.
Depth of parafine and oil, 2.5 cm.

An equal amount of paraffine in a similar tube, however, reduced the band of undecolorized medium to 1 cm. in sixteen hours. In a comparison of these three conditions where boiling was used as a means of immediate decolorization followed by rapid cooling and incubation at 37°C., the measurements of the blue band indicated (in centimeters) in table 6 were made.

An analogous comparison of paraffine and vaseline of approximately equal melting points (50°C.) and boiling points (about 300°C.) showed vaseline to be superior even to paraffine. Table 7 shows the actual measurements in centimeters (alkaline methylene blue agar 7 cm. deep).

The recession of the band under paraffine and vaseline was not peculiar to this particular experiment; it has frequently been

seen, but never with oil nor in media without covering except under the influence of light. It has a logical explanation, I believe in the assumption that a certain limited amount of oxygen and carbon dioxide are absorbed from the seal, thus accounting for the band observed, but failure or reduction in the supply coming through when the seal hardens permits the loss by diffusion from the lower surface of the blue band of these gases into the deeper layers of medium and their dilution thereby to a concentration insufficient to recolor the dye.

TABLE 7

A comparison of paraffine and vaseline as seals against the recoloration of alkaline methylene blue agar

	HOURS			
	1.5	4	24	50
	cm.	cm.	cm.	cm.
No seal	0.7	1.1	2.0	2.3
Paraffine	0.6	0.7	1.3	0.7
Vaseline	0.2	0.2	0.3	0.0

Tests with the liquid solution also indicate the great superiority of paraffine wax and especially vaseline over mineral oil as a means of protecting decolorized methylene blue solutions from recoloration. These compare favorably with the mechanical seals such as the marble in a constricted tube, or the cover glass in either plain or constricted tube, or sand in a Smith fermentation tube, under all of which the dye may remain water clear for days.

The efficacy of these seals is not a matter of boiling points since mineral oil has approximately the same boiling point as paraffine. Furthermore, the lower boiling hydrocarbons such as xylol (137° to 140°C.) and heptane (95° to 100°C.) are even less efficacious than mineral oil. Viscosity and consistency seem to be the essential elements; perhaps the ease with which vaseline clings to the glass on hardening explains its superiority over paraffine. The liquid oils probably permit the return of absorbed gases but more particularly operate through convection currents

which transfer gases absorbed at the air surface to the oil-medium surface. Such convection currents are absent, of course, in the solid waxes and semisolid greases.

These researches would be barren were we not able to affirm the parallelism of cultural studies. As a matter of fact, dilution experiments with such organisms as *B. tetani*, *B. Welchii*, *B. sporogenes* and others confirm the great value of paraffine and vaseline as compared with liquid mineral oil. *B. tetani* absolutely failed to grow under either xylol or heptane, gave only delayed turbidity under mineral oil and vigorous early gas production under paraffine. Yet none of these is germicidal for *B. tetani* as shown by successful growth under the marble in a constricted tube of glucose broth covered with them.

The great objections to vaseline and paraffine are their messiness and the fact that they do not provide sufficient variety of oxygen pressures in the medium to meet the possible requirements of different organisms.

Boiling as a means of oxygen tension reduction is sometimes used in methods where the air chamber is sealed either by fusion of the glass outlet or by mechanical devices such as valves, cocks, etc. Either method is satisfactory from the standpoint of the persistence of the decolorized state of methylene blue but is obviously superfluous for deep cultures and is inapplicable to surface cultures for reasons easily apparent.

Evacuation by water pump to 58 cm. mercurial pressure with immediate sealing of a constricted portion of the tube has never sufficed to decolorize methylene blue 1:100,000 either with 2 per cent glucose and N/500 NaOH or 2 per cent agar and N/100 NaOH at ordinary room temperature in our hands. Nor have we ever been able to secure surface growths of such obligate anaerobes as *B. Welchii*, *B. tetani*, *B. oedematis* and *B. botulinus* on the surface of solidified glucose agar by this means alone. We are therefore forced to conclude that evacuation by water pumps of such efficiency is of relatively little value alone as a method of securing anaerobiosis. Higher evacuation might yield more successful results.

The literature is notably deficient in accurate data on the oxygen tension limits of anaerobic bacteria determined by vacuumetric methods. The limit of 35 cm. Hg pressure for the *vibrion septique* set by Rosenthal (1906) in liquid media is of doubtful value when viewed in the light of the recent observations of Harris (1919) on the apparently high tolerance of *B. sporogenes* for oxygen in liquid cultures as compared with agar slopes.

While we have tested the effect of inert gases in the case of hydrogen and carbon dioxide, it is scarcely fair to consider the results as necessarily corresponding to those obtained by cultural methods where we conceive the action to be primarily physical, since with methylene blue solutions we may also have chemical reactions. Hydrogen, indeed, did decolorize cold solutions of 0.5 per cent glucose with N/500 NaOH and 1:100,000 methylene blue slowly, and hot solutions already decolorized remained so during fifteen minutes of active ebullition by hydrogen from a Kipp generator loaded with zinc and sulfuric acid and purified by passage through 10 per cent $PhNO_3$ and alkaline pyrogallol. Furthermore when sealed the hot solution remained decolorized till opened on the fourth day while the cold solution showed only a trace of color while sealed.

Passage of commercial carbon dioxide through similar solutions of the dye for one hour failed to decolorize the cold solution although the hot solution remained decolorized during this part of the experiment but soon regained the blue color after sealing. Reheating these solutions failed to decolorize either of them, without further addition of alkali. Here is a situation, which, barring the known inhibitive action due to improper acidity for certain organisms, might yield satisfactory results in the case of certain others, as Pasteur found, without permitting a satisfactory degree of alkalinity for the decolorization of methylene blue.

Coming now to the matter of chemical reduction of oxygen tension, we conceive that in so far as regards reducing agents *in the medium*, they have been sufficiently dealt with already, and the necessity of recognizing limitations of time and space

reduces this discussion, in so far as it relates to chemical reduction by means of an agent *in the air* chamber, to the most valuable agent with which we have to deal, i.e., alkaline-pyrogallol.

Inasmuch as this reagent finds a legitimate use only in connection with attempts to secure surface colonies on solid media, it is to this that we have limited our attention. Buchner's (1888) original technic involving the use of a small tube containing slanted culture medium (2 per cent agar, methylene blue 1:100,000, N/100 NaOH) in a longer one containing the usual alkaline pyrogallol mixture gave satisfactory results when the cotton stopper was left out of the inner tube, but otherwise did not, unless the tube were inverted. Decolorization begins, as does growth of obligate anaerobes on suitable media, in the thinnest portion of the slant. In Wright's (1901) modification, which involves the saturation of the plug with the mixture, sealing by rubber stopper, and inversion, even better results were obtained. In either case partial decolorization appeared at the surface in less than twenty-four hours at 37°C. and continued to completion within two to three days. Both these methods, but notably the last, have given quite satisfactory results in the surface cultivation of *B. tetani*, *B. welchii*, *B. sporogenes*, *B. chauveauii*, *B. botulinus*, and many unidentified anaerobes. Wright's method has been relied upon largely for repeated surface colony isolation of the writer's cultures.

Two plating methods for surface culture have been studied in connection with the methylene blue test, namely Lentz's (1910) pyrogallic acid saturated cardboard, and McLeod's (1913) divided plate. Both showed the dye-agar decolorized at 37°C. within eighteen hours. The latter method has refused consistently however to yield us surface colonies of well known anaerobes on either blood or glucose agar identical with that used in controls by Wright's method. Dr. Oskar Klotz at the University of Pittsburg has stated his belief that aromatic substances in the clay are sufficiently germicidal to explain the failure of bacterial growth and has overcome the difficulty through the use of a special cement containing paraffine and some other substances. At any rate, McCleod's device seems constructed

according to our best conceptions of the requirements. In contrast, the method of Lentz is open to the technical objection that considerable absorption may occur before the seal is completed. We have not tested the latter sufficiently from a bacteriological standpoint to justify any conclusion as to its real worth. The dye test points to its suitability.

In conclusion it is suggested that further studies are required to show exactly to what degree of oxygen tension reduction the decolorization of methylene blue under stated conditions corresponds. Harvey's (1919) recent demonstration of the direct relation between oxygen content and time of decolorization of methylene blue in Schardinger's test and the interesting parallelism between methylene blue reduction and luciferin formation (1920) is significant in this regard, but one must not overlook the great importance of the hydrogen ion concentration of the test solution, and the effect upon this of atmospheric carbon dioxide.

Similar studies are equally needed in the investigation of the oxygen relations of obligate anaerobic bacteria.

SUMMARY

This paper reviews the literature on chemical criteria of anaerobiosis, and undertakes a critical experimental survey of the subject in so far as it relates to the use of methylene blue for this purpose. The mechanism of the decolorization of methylene blue is studied in detail and shown to depend upon the interaction of alkali and certain organic substances, notably carbohydrates. A correspondence between Fehling's test and the decolorization of methylene blue in alkaline solutions of various carbohydrates is pointed out.

It is shown that while there is a direct relation between the amount of dye decolorized by heating and the amounts of alkali and glucose, there is an inverse relation between the last two factors in the test, so that an increase in one permits a decrease in the other for the same result.

Carbon dioxide, as well as oxygen, is shown to be an effective factor in the recoloration of decolorized methylene blue.

Various methods of anaerobiosis are viewed critically in the light of experimental tests with carefully balanced solutions of alkaline glucose methylene blue in comparison with cultural tests with *B. Welchii*, *B. tetani*, *B. botulinus*, and other obligate anaerobes.

A detailed study of the decolorization of methylene blue by plant and animal tissues is described, showing the important role of adsorption as a means of decolorization by these and other porous substances. The extraction from plant and animal tissues of reducing substances for methylene blue, active in alkaline solution, is described.

The efficacy of deep culture methods for anaerobes is shown while the inefficacy of insoluble liquid (i.e., oil) seals is contrasted with the reliability of semi-solid waxes and greases, and that of mechanical seals.

The short-comings of certain methods of surface culture of obligative anaerobes are exposed and the value of a modification of Wright's method upheld by these studies.

Finally, the desirability is indicated of determining exactly to what degree of oxygen tension reduction the decolorization of methylene blue corresponds, and whether decolorization occurs at a definite hydrogen ion concentration irrespective of the sugar content of the solution.

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Sanitary Research Laboratories, Massachusetts Institute of Technology

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The wide use of litmus milk as a culture medium, due to its great value in the differentiation of bacteria, has led to the development of various casein media as substitutes. Canned milk has also been used with varying degrees of success. This paper presents a formula which insures a standard product.

Present practice requires the purchase of milk, whole or skimmed. In the former case the fat has to be removed. When the milk is finally prepared it is often found to be too acid and readjustment is required. After such a readjustment is made the effect of sterilization of the milk is often detrimental. But what is more important is the varying color of the milk with every batch. It was with this fact in view that the present formula was developed.

PREPARATION OF THE INDICATOR

1. The dye should be a blue azolitmin, as free as possible from the red dye. This may be prepared by extracting litmus cubes with boiling distilled water and decanting off the clear solution. The liquid is evaporated to a thin paste over a free flame and treated with an excess of glacial acetic acid. The evaporation is now carried to dryness on a water bath. A quantity of 95 per cent alcohol is added and the whole transferred to a filter. The residue is washed several times with alcohol. The filtrate is discarded. Should the residue be left reddish a drop or two of sodium hydroxide solution will restore

its color. The residue is dissolved in water. This is evaporated to dryness, first over a free flame and finally on the water bath. The yield is about one twenty-fifth of the original weight of the cubes. (Azolitmin may be purchased from certain dealers.)

2. A satisfactory powder may be prepared by extracting the cubes with hot water, filtering and evaporating the filtrate to dryness. The residue is treated with 95 per cent alcohol overnight. In the morning the alcohol is decanted off and the residue dried on the water bath.

THE MILK

Skimmed milk powder is purchased from a bakers' supply company or grocer. Care should be taken to see that it is clean. Quantities in small containers are more satisfactory than bulk purchases.

The litmus powder so prepared is mixed with the milk powder. In the case of the litmus powder prepared by the first method (or purchased azolitmin powder) one part of the dye is added to 52.7 parts of the skimmed milk powder. When the dye is prepared by the second method one part of the dye powder is added to 49.6 parts of the milk powder.

The resulting mixture is dissolved in distilled water in the proportion of one part of the litmus milk powder to 9.5 parts of water.

Care should be taken in the mixing that the litmus powder is well ground and well dispersed in the milk powder.

All media made in this way have been sterilized perfectly in the Arnold Sterilizer. When autoclaved at 15 pounds for five minutes the medium resumes its true color on cooling. The mixed powder has kept perfectly in a glass stoppered bottle for two years.

Cultures of *Bact. coli*, *B. Welchii*, *Bact. acidi-lactici*, *Bact. alcaligenes*, *Pseudomonas fluorescens*, *B. mesentericus-vulgatus*, *Staph. pyogenes-albus*, and *Proteus vulgaris* all grew well and gave typical reactions in the normal time.

BACTERIA CONCERNED IN THE RIPENING OF CORN SILAGE

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The production of silage on the farm may be regarded as a means of conserving valuable food material. During the process of fermentation which takes place in the silo the fresh fodder is so altered as to prevent spoilage and to conserve its food value. Experience as well as experiment has shown that a highly nutritious and palatable food is thus made available during the winter months, when fresh fodder cannot be obtained.

The authors make no claim to have solved all the problems connected with the fermentation of silage. Many details must remain for future investigation and all that can be claimed is progress. Hunter and Bushnell and Sherman lay stress on the activity of lactobacilli in the ripening of silage and the present work confirms the findings of these authors, namely that lactobacilli are important factors, but that they probably reach the maximum of their activity during late stages of fermentation.

Opportunity presented itself to investigate silage from three silos. Two of these are constructed of wood with concrete foundation, while the third is concrete throughout. The investigation, as might be anticipated, shows that although the fermentative process in general is similar in different silos, it is by no means identical. That is to say, after a definite lapse of time the silage of one silo may be fermented in larger measure than that in another silo. Furthermore, the same stage of fermentation does not necessarily obtain throughout the same silo. No relation to the difference in construction could however be detected.

The silage in the three silos was removed from the top as it was required as food for cattle. Therefore the samples obtained for investigation had not only fermented during progressively longer periods of time, but since the surface layers were periodically removed samples had to be procured from increasing depths. It is conceivable, therefore that anaerobic fermentation becomes more pronounced as silage ripens.

METHODS

Samples were taken from the silos in November, January, February, March, April, May and June. A few days elapsed between sampling the different silos in order not to crowd the work but this short interval probably made no material difference in the relative stage of fermentation. Ten grams of each sample were infused with 50 cc. of cold physiologic salt solution. From this infusion stains were prepared and inoculations made in milk with addition of brom-cresol-purple as indicator, into flasks and fermentation tubes containing 2 per cent glucose broth, and on agar. The last medium was used for surface and stab cultures. The infusion of silage was titrated with N/20 NaOH with phenolphthalein as indicator and then together with the inoculated media was incubated at 37°C.

RESULTS

Stains from the silage infusion showed enormous numbers of microörganisms. No attempt was made to enumerate them or separate the types, but the predominating types were noted and cultivated. Yeast cells were invariably present and frequently formed a solid film on the surface of the incubated infusion. The acidity of this infusion was never more than 3 per cent normal, but ti is probable that the salt solution did not dissolve all the acid contained in the silage. If the juice had been pressed from the silage and then diluted five times its volume with salt solution the acidity would probably have proved higher than the values obtained. After 24 hours incubation of the silage infusion the acidity rose to about three

times the original amount, but after forty-eight hours a marked decrease of acidity was observed coincident with the development of a heavy film consisting of yeast cells.

A bacillus of the colon-aerogenes group was present in large numbers, but only in smears prepared from silage during early stages of fermentation. During late stages the bacillus may still have been present, but if so was numerically insignificant. Streptococci and lactobacilli were always present in early as well as in late samples. The streptococci were readily isolated by plating, but the lactobacilli did not grow readily on ordinary media and were isolated by the method employed by Heineman and Hefferan, namely by repeated transfers through milk and final plating on whey agar.

The streptococci isolated varied somewhat morphologically. In some cases the diplococcus form was predominant, while in others chain formation was observed. However, the diplococcus form was found chiefly in early samples of silage, while chain formation was abundant in later samples. Chains were prominent also in stock cultures prepared from the isolated streptococci, but the short form reappeared when fresh subcultures were prepared. The fact has been repeatedly recorded, that during the most active period of growth, streptococci, especially saprophytic ones, appear chiefly in diplococcus form, while after the maximum growth has passed chain formation becomes more evident. The size of individual cells of streptococci confirms the assumption that they are more active in early silage than in the ripened product. From early samples the cells were uniformly small and in later samples they were large and frequently appeared swollen. These abnormal forms disappeared when young generations grew on inoculated media.

The amount of acid formed in glucose broth by the streptococci was determined by titration with N/20 NaOH. The quantity thus determined never exceeded 5 per cent normal acid in five days. Milk was readily coagulated by most strains of streptococci isolated, in some cases within twenty-four hours.

The lactobacilli observed occurred singly, showed granular staining with methylene-blue in many instances and were rather difficult to isolate. They grew slowly on media containing 2

per cent glucose and the colonies formed on whey agar were always very small. However, from the latest samples a streptobacillus was isolated which grew with relative abundance on glucose media and produced up to 11.5 per cent acid in glucose broth in six to seven days. This bacillus appeared in long filaments composed of rather short bacilli. The acid produced consisted chiefly of lactic acid. It should be emphasized that this strepto-bacillus was never observed in early samples, although it probably was present in small numbers.

Milk inoculated with two loopfuls of silage infusion coagulated promptly, usually within twenty-four hours and when inoculated with the infusion of early samples considerable amounts of gas were produced. In some instances the gas formation was so vigorous as to break up the coagulum. As ripening of the silage progressed the amount of gas formed in milk decreased and finally in the last stages of fermentation no gas appeared. Stains from the coagulated milk, prepared after twenty-four hours incubation, showed that in early stages organisms of the colon-aerogenes group were present in abundance, accompanied by large numbers of streptococci, but as the ripening process proceeded streptococci became more prominent and finally were present practically in pure culture. The milk cultures were not incubated for a sufficient length of time to permit lactobacilli to supersede streptococci, excepting when lactobacilli were searched for.

In a recent paper Gorini calls attention to the presence of butyric acid bacilli in silage and warns of the possibility of their influence on the product. Since anaerobic conditions obtain, at least in deep layers of silage, and since the temperature may be relatively high, conditions for the growth of butyric acid bacilli are not unfavorable. Gorini suggests that the temperature be not permitted to go beyond 50°C. nor below 30°C. in order to favor the growth of lactic ferments (lactobacilli and streptococci) and thus antagonize butyric acid bacilli. In our limited investigation no evidence of the presence of butyric acid bacilli was observed, although they may have been present in small numbers. Several anaerobic cultures prepared from the silage infusion invariably gave a growth of streptococci, practically in pure culture.

DISCUSSION

As stated before the investigation reported in this paper does not warrant very definite conclusions and the following suggestions are offered tentatively. It is a well known fact that the groups of colon-aerogenes bacilli, of streptococci and of lactobacilli are widely distributed in nature and especially on fodder. Lacto-bacilli were found by Heineman and Hefferan in cornmeal and by Sherman on fresh corn leaves. The presence of these groups in silage may therefore be considered as established. This condition recalls observations on the so-called normal souring of milk and the activity of streptococci and lactobacilli during cheese ripening. Assuming that the colon-aerogenes group is present in fresh silage a limited growth of these organisms would produce an initial fermentation with acid and gas as the chief products. This assumption is supported by the fact that considerable pressure is developed during the ripening of silage and by our observation that gas is formed in abundance in milk cultures prepared from samples of silage in early stages of ripening. The colon-aerogenes group is soon suppressed by the streptococci. Acid is the chief product of this group. Milk cultures showed a progressive decrease of gas formation a fact which indicates the gradual suppression of the colon-aerogenes group. Streptococci are then crowded out by lactobacilli, but probably never disappear entirely as they could be isolated readily from silage in its last stages of fermentation. It is suggested that at least two groups of lactobacilli are active. The first group is of the slow-growing type, the bacilli occurring singly and showing granular staining with methylene-blue. The second group of lactobacilli is of the more rapid-growing order and is readily isolated from later stages of fermenting silage.

Wyant inoculated corn silage experimentally with cultures of *Streptococcus lacticus* and *Lactobacillus bulgaricus* and obtained a product which according to the author's description resembled normal silage. However, after a period of four to five weeks lactobacilli were not isolated, while streptococci were recovered without difficulty. This shows that the silage had not passed

beyond the second phase of fermentation. According to the investigation reported in this paper the third phase of fermentation, when lactobacilli are active, does not occur during the first weeks of the ripening process.

The investigation came to a conclusion because the silage was practically exhausted in the silos whose product was available. The remaining silage contained considerable quantity of acid and the owners of the silos hesitated to use this remnant for fodder. It does not seem, however, that the acidity was great enough to cause injury to the cattle. Fair samples could not be obtained from these remnants.

It has been stated that stains from early samples of silage showed the presence of large numbers of microörganisms of various types. During the progress of the work the number of types was gradually reduced and the microscopic picture of stains from the last stages of ripening differed materially from that obtained from early stages, inasmuch as streptococci and lactobacilli were clearly predominant and other forms had disappeared in very large measure. No doubt the result is explained by the accumulation of acid in the final product. The disappearing bacteria may influence the ripening process by consuming oxygen and thus create favorable conditions for facultative anaerobes, such as streptococci and lactobacilli.

CONCLUSION

The authors as a result of this investigation offer as a tentative hypothesis of the ripening process of corn silage the following: The fermentation consists of three phases brought about by three groups of bacteria. The initial phase is of short duration and is caused by members of the colon-aerogenes group of bacteria. It is accompanied by acid and gas formation. The second phase is carried on by streptococci and is accompanied by moderate acid formation. The third phase is the result of the activity of lactobacilli. The third phase may possibly be subdivided into minor phases owing to the presence of different strains of lactobacilli. It should be added that the assumption

of some investigators that the first process of silage ripening is a respiratory process (Babcock and Russell) is not excluded by the hypothesis advanced and does not interfere with it. Furthermore, mention should be made of the fact that at no time was growth of yeasts in the silage observed, although yeast cells were invariably present. Growth of yeast was observed only when an extract of silage was incubated at 37°C. There is therefore in this investigation no indication of an alcoholic or acetic acid fermentation as suggested by Esten and Mason.

It should be emphasized that the assumption of different phases of silage fermentation does not mean that the phases are distinctly separated, or that a period of rest occurs when one phase passes into the next one, or finally that the same phase is operative throughout the whole quantity of silage. The authors are inclined to the opinion that conditions of ripening are not the same in different parts of the silage and that unless samples are taken from definite regions, if this were possible, the progress of ripening would appear somewhat obscured. It is suggested further that the process of ripening may differ in kind and in degree in different silos, owing to variations in construction, in moisture content, and to climatic conditions. However, the fundamental principles and the groups of organisms active in the fermentation of silage are similar under a variety of conditions.

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SOME ATYPICAL COLON-AEROGENES FORMS ISOLATED FROM NATURAL WATERS

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Attempts to bring cultures isolated from routine water samples in the laboratory of the Illinois State Water Survey within the tentative classification of the committee of the American Public Health Association (1917) reveal certain inadequacies of the scheme, some of which have been previously noted in the literature without emphasis.

ANOMALOUS METHYL RED–VOGES-PROSKAUER REACTIONS

Correlation of the methyl red reaction with the Voges-Proskauer reaction has been adjudged almost complete for low ratio organisms; but for high ratio types, a very considerable number of exceptions have been noted.

Berrier, McCrady and Lafreniere (1916), applying these tests to 450 organisms isolated from feces, city sewage and grains, found the Voges-Proskauer and methyl red tests to agree completely with the generally accepted standard tests for *Bact. coli* organisms when applied to 197 strains from human feces, except in one instance. Applied to grain and sewage cultures the correlation was found in 80 per cent of the cases.

Levine (1916) cites a small group of organisms isolated from soil, resembling *Bact. acrogenes* with respect to gas formation from various carbohydrates, etc., which did not give the Voges-Proskauer reaction and were neutral to methyl red after three days' incubation at body temperature. Some did not give the Voges-Proskauer reaction and were not alkaline to methyl red until the fifth or seventh day of incubation. These resemble closely a form described by MacConkey, who records the Voges-Proskauer reaction as positive or negative.

The strains described by Johnson and Levine (1917) from soil include four methyl red neutral, Voges-Proskauer positive; 13 methyl red neutral, Voges-Proskauer negative; and 2 methyl red negative, Voges-Proskauer negative. The temperature and period of incubation differ from those now current, but concordant results recorded in the same paper are cited by Rogers, Clark and Lubs (1918) and by Winslow, Kligler and Rothberg (1919).

Burton and Rettger (1917) report the biometric method inapplicable to the colon-aerogenes group on account of the marked variability of organisms of the high ratio type with respect to the methyl red test in Clark and Lubs medium (1915) as well as in others employed. While variability with regard to the Voges-Proskauer reaction was noticeable, it was less frequent than in the methyl red test. They refer to two cases found by Rogers, Clark and Davis (1914) and Rogers, Clark and Evans (1915) where an organism had apparently altered its gas ratio profoundly, explained by the authors as possibly due to an error in lettering apparatus, remarking that this is undoubtedly the safer explanation, but that in view of the results of themselves and others in this field variability must also be reckoned with. In explaining variability of high ratio organisms they suggest that metabolism may take two courses, not necessarily parallel or of equal rate: the fermentations may proceed irregularly and yield equivocal results even when the Witte peptone broth of Clark and Lubs is used. With the same strain there may be rapid exhaustion of sugar, heavy growth, large gas volume, high gas ratio and low acidity; or incomplete sugar utilization, small gas volume, low gas ratio, and high acidity. If neither factor predominates it would be possible to have an organism capable of giving the Voges-Proskauer reaction and an acid reaction with methyl red. The authors cite one strain which became persistently methyl red positive and Voges-Proskauer positive.

Burton (1916) in his thesis, of which Burton and Rettger present the summary, instances 50 strains from sources, mostly unpolluted, which gave conflicting or variable methyl red and Voges-Proskauer reactions.

Miss Bixby (1918) reports 6 strains from waters which are methyl red and Voges-Proskauer positive; and 1 which is negative in both reactions.

Levine (1918) included in the aerogenes-cloacae group all strains which gave the Voges-Proskauer reaction, "practically always alkaline to methyl red," and 10 cultures which fermented starch with gas formation but did not react typically for the Voges-Proskauer nor the methyl red tests. Of the 151 organisms 142 were from soil, 9 from sewage.

Winslow and Cohen (1918) report perfect correlation between the methyl red and Voges-Proskauer reactions for 53 strains in a total of 54 isolated from polluted, unpolluted and stored water. Winslow, Kligler and Rothberg (1919) speak of their series of high ratio cultures as including 8 of the *Bact. cloacae* and 23 of the *Bact. aerogenes* type, all but 1 alkaline to methyl red and all but 8 Voges-Proskauer positive.

Rettger and Chen (1919) report an "almost perfect correlation between the two types" in the synthetic as well as in the Witte's peptone medium (not in Difco) when the incubation period was prolonged to five days. We have seen only the authors' abstract of this paper.

INTERPRETATION OF ADONITOL REACTIONS

The adonitol positive reaction has been considered discriminative of *Bact. aerogenes* of fecal origin, and is so rated in the rather diagrammatic scheme of the committee of the American Public Health Association (1917). But Rogers (1918) considers that while *Bact. aerogenes* isolated from feces is adonitol positive, it does not necessarily follow that all waterborne *Bact. aerogenes* with this character are therefore derived from immediate fecal sources.

Rogers, Clark and Lubs (1918) isolated aerogenes strains from stools of but three out of eighteen persons; all of the 46 strains were adonitol positive; but of the low ratio cultures from similar sources 17 were likewise adonitol positive (12.98 per cent).

Darling (1919) cites numerous references in confirmation of his findings: of 113 coli-like cultures isolated from feces of man

and of animals none were *Bact. aerogenes*. To his references may be added Hulton (1916), Stokes (1919) and Rettger and Chen (1919) who encountered no organisms of the *Bact. aerogenes* type in 173 cultures isolated from feces.

Far from being a specific reagent for members of the aerogenes-cloacae group, adonitol probably deserves a place not much superior to dulcitol as a reagent of rather dubious import in discriminating members of the low ratio group. Winslow, Kligler and Rothberg (1919) summarize the earlier work of Kligler (1914) and Levine (1918) in tabular form, to which we add in parentheses the less usual reactions of these and the high ratio types:

	SUCROSE	SALICIN	DUCITOL	ADONITOL
Bact. aerogenes	+	+	+−	+−
Bact. cloacae	+	+	+−	+−
Bact. neapolitanus	+	−	−(+)	−(+)
Bact. communior	+	−	+(−)	−
Bact. coli	−	+	+(−)	−
Bact. acidi-lactici	*	*	−	+(−)

Adonitol is important as being sometimes included in the list of sugars, etc., fermented by *Bact. aerogenes*, which Winslow and his co-workers consider as perhaps the most primitive of the colon-typhoid group, and of the highest fermentative power. As such it is least significant as an indicator of fecal pollution. The significance of the so-called "fecal aerogenes type" in waters is probably slight.

DEPARTURES FROM STANDARD METHODS

Because of the shortage of Witte's peptone and the impossibility of obtaining material for the synthetic medium of Clark and Lubs (1917) for testing the methyl red reaction, Difco peptone was substituted in the initial determinations. Preliminary tests on pure cultures with 0.75 per cent Difco, properly buffered, and incubated at 30°C. for two days, gave results identical with those of the same cultures in the standard Witte broth. As the number of strains isolated increased, tests were repeated with

newly found cultures, using 0.5 per cent, 0.75 per cent and 1 per cent Difco in comparison with the standard methyl red broth. While the Difco medium, with whatever concentration used, is not equivalent to the standard broth, the reversion of acid reaction with definite concentrations of the substituted peptone with a definite buffer reaction presents phenomena affording a basis for division of cultures into provisional groups. From the strains thus segregated some were selected for study with standard media.

Koser (1918) proposed the use of a medium containing no nitrogen except in the form of uric acid for the discrimination of colon and aerogenes forms, reporting the results with 74 strains of *Bact. coli* and 50 of *Bact. aerogenes:* the former showed no growth; the latter grew well. Rettger and Chen found that, with few exceptions, among the colon strains from soils the uric acid test gave very satisfactory correlation with the other reactions when necessary precautions were taken. Their culture comprised 447 strains of the cloacae-aerogenes group and 20 of the colon type from unpolluted soils, and 173 strains from feces of men and of animals.

To test the validity of this reaction as a criterion of high and low ratio organisms, we have arranged our cultures isolated from waters with reference to their uric acid reactions, for later comparison with the arrangement of specially studied strains grouped according to their methyl red and Voges-Proskauer reactions.

STUDY OF CULTURES ISOLATED FROM WATERS

In the course of this work 392 cultures were isolated which gave gas in lactose broth, more or less characteristic colonies on Endo's medium, and usually gas in lactose broth after transfer from the endo medium. They were tested as to their reaction in Difco methyl red broths after two days; with adonitol and with uric acid broth. The first series comprised 233, the second, 159 strains. The results are summarized in table 1. Arranged according to lactose fermentation, adonitol and uric acid reactions, they fall into 19 provisional groups to which are assigned reference numbers of purely temporary value.

The first six groups of strains (uric acid positive adonitol negative) may be regarded as varying about the third, which is typical "non-fecal aerogenes." Those grouped under numbers seven to ten inclusive may be thought of as variants of the so-called "fecal aerogenes type" (number eight uric acid positive, adonitol positive). Those grouped under numbers eleven to

TABLE 1

Provisional arrangement of strains isolated from water

REFERENCE NUMBER	LACTOSE BROTH	ENDO'S MEDIUM	LACTOSE BROTH	METHYL RED DIFCO		ADONITOL	URIC ACID	SPORES	NUMBER OF SERIES		STRAINS TOTAL
				0.5 percent	0.75 percent				1	2	
1	+	+	−	−	−	−	+	−	16		16
2	+	+	−	+	−	−	+	−	2		2
3	+	+	+	−	−	−	+	−	19	6	25
4	+	+	+	−	−	−	+	+	1		1
5	+	+	+	+	−	−	+	−	4	4	8
6	+	+	+	+	+	−	+	−	6	3	9
7	+	+	−	−	−	+	+	−	4		4
8	+	+	+	−	−	+	+	−	27	6	33
9	+	+	+	+	−	+	+	−	11	5	16
10	+	+	+	+	+	+	+	−	16	14	30
11	+	+	+	+	+	+	−	−	15	5	20
12	+	+	+	+	−	+	−	−	3		3
13	+	+	+	+	+	−	−	−	84	113	197
14	+	+	+	+	−	−	−	−	5		5
15	+	+	+	+	+	−	−	+	1		1
16	+	+	+	−	−	−	−	−	2		2
16a	+	+	+	−	−	−	−	+		1	1
17	+	+	−	+	+	−	−	−	8	2	10
18	+	+	?	+	+	−	−	+	2		2
19	+	+	−	−	−	−	−	−	7		7
									233	159	392

sixteen inclusive (uric acid negative, adonitol positive or negative) may be variants of number 13—typical *Bact. coli.*

From numbers 1 to 6 and from 7 to 10 there is progressive increase in net acidity to the limit of pH 5.8; from 11 to 16 there is a decrease in acid formation, and from 17 to 19 there is irregularity in acidity and in gas formation in lactose. Five spore-bearing forms were isolated, which are fully described in a forthcoming paper.

The second series represents strains which passed through enrichment processes whenever they showed delayed reaction with media. Perhaps this may have somewhat diminished the number of forms varying about the three types. In the course of this treatment the purity of our strains was assured.

There are differences in reactions of strains from a single source or from one sample of water. Nine of the samples were from 75-foot tubular wells in the same stratum: two were from a group of wells furnishing a city supply, and the remaining seven were from a single nearby well. Table 2 shows the numbers of strains

TABLE 2

Strains isolated from one source

REFERENCE NUMBER	NUMBER OF STRAINS	REACTIONS WITH	
		Uric acid	Adonitol
3	6	+	−
6	2	+	+
8	9	+	+
9	3	+	+
10	2	+	+
11	7	−	+
13	12	−	−
17	2	−	−
19	1	−	−
	44		

assigned to each provisional group. As many as 7 strains, distributed among 6 groups, were found in a single sample. Of the 44 strains assigned to 9 groups, 22 are uric acid positive; 14 uric acid positive, adonitol positive; and 14 fall into the transition groups 5, 9, 10, and 11. Eight vary about "non-fecal," and 14 about "fecal aerogenes;" 19 about the *Bact. coli* type, and 3 give slow lactose fermentation. Other instances of varying strains from a single sample can be found in table 3.

SPECIAL STUDY OF STRAINS IN TRANSITION GROUPS

Thirty-five strains, mostly from the second series, were tested with a number of sugars, alcohols, etc. (In these tests both a

TABLE 3

Reactions of strains in transition groups

REFERENCE NUMBER	SOCIETY NUMBER	URIC ACID	METHYL RED	VOGES-PROSKAUER	SALICIN	DULCITOL	ADONITOL	
4	121.1112033	+	var.	+	−	−	−	
5a	222.1112031	+	+	+	−	−	−	
5b	222.1132031	+	+	−	−	−	−	Raffinose +
6a	222.1132031	+	+	−	+	+	−	*Bact. coli*
6a	222.113 031	+	+			+	−	*Bact. coli*
6b	222.1112031	+	+	−	+	+	−	*Bact. aerogenes*
9a	222.1112031	+	−	+	+	+	+	*Bact. aerogenes*
9b	222.111 031	+	−			−	+	*Bact. aerogenes*?
9c	222.1112031	+	+	−	−	+	+	*Bact. communior*?
9c	222.111 031	+	+			+	+	*Bact. communior*?
9c	222.1112031	+	+	+	+	+	+	*Bact. aerogenes*
10a	222.1112031	+	+	−	+	−	+	*Bact. aerogenes*? Raff.—
10b	222.1112031	+	+	+	+	−	+	*Bact. aerogenes*
10b	222.1112031	+	+	+		−	+	*Bact. aerogenes*?
10b	222.1112031	+	+	+	+	−	+	*Bact. aerogenes*
10b	222.1112031	+	+	+	+	−	+	*Bact. aerogenes*
10b	222.1112031	+	+	var.	+	−	+	*Bact. aerogenes*
10b	222.1112031	+	+	+	+	−	+	*Bact. aerogenes*
10b	222.1112031	+	−	−		−	+	*Bact. aerogenes*?
10c	221.1112031	+	+	−	+	−	+	*Bact. aerogenes*?
10d	222.1112033	+	+	−	+	+	+	*Bact. aerogenes*
10d	222.1112033	+	+	−	+	+	+	*Bact. aerogenes*
10d	222.1112033	+	+	−	+	+	+	*Bact. aerogenes*
10d	222.1112033	+	+	−	+	+	+	*Bact. aerogenes*
10d	222.1112033	+	var.	−	−	+	+	*Bact. communior*?
11a	222.1112031	−	+	−	−	−	+	*Bact. communior*?
11c	222.1112033	−	+	−	+	+	+	?
11c	222.1112033	−	+	−	+	+	+	?
11b	222.1132031	−	+	−	+	−	+	*B. coli*?
11b	222.1132031	−	+	−	+	−	+	*B. coli*?
15	121.1112011	−	var.	−	−	−	−	
16a	121.1113011	−	−	+	−	−	−	
17	222.333 033	−	+			−	−	
18a	121.1332032	−	var.	+	−	−	−	
18b	121.1332033	−	−	+	−	−	−	

0.2 per cent and the standard 1 per cent sugar, alcohol and starch broths were used with identical results.) Some of the strains were lost before the series of tests was complete. It will

be noted that members of the same provisional group show considerable divergence in their ability to react with sugars, etc., and with gelatine. If these variations are made the basis of subdivision, the number of provisional groups is increased, as indicated by literal suffixes to the reference numbers in table 3. The cultures are arranged as before, primarily with regard to their lactose, uric acid and adonitol reactions, then with reference to sucrose, dulcitol and glycerol.

For the sake of conciseness the reactions of strains are represented so far as is possible by the numerical scheme of the Society's chart, with the addition of methyl red and Voges-Proskauer reactions in standard media and a few other reactions. Maltose and mannitol were fermented with gas formation by all strains except 17, 18a and 18b. Starch was attacked with gas formation by but 2 strains, and those were sporeformers. Gelatine was liquefied by 10c and by 5 sporeformers. Raffinose and sucrose reactions were of like signs for all save 5b and 10a.

The inclusion of sporebearing forms in this table is anticipated by the work of Loehnis and Smith (1916), who state that a single species (particularly *Azotobacter*) may pass through as many as 12 to 14 distinct morphological forms in its life cycle, including spores. Kellermann and Scales (1916), in a preliminary report on the life cycle of *Bact. coli*, studied 12 strains from widely different sources which were found to produce all the types described by Loehnis and Smith except spores. Burton and Rettger report 9 occurrences of a form differing chiefly from our No. 15 in that theirs is Gram positive. Meyer (1918) and Ewing (1919) isolated from waters a spore-bearing, lactose-fermenting, acid-forming bacillus which seems identical with our 16b. Itano and Neill (1919) found sporeformation by *B. subtilis* to be a function of temperature and hydrogen ion concentration: it is possible that unfavorable environment may lead to sporeformation by members of the colon-aerogenes group.

Methyl red reactions in different concentrations of Difco peptone varied upon repetition. The strains grouped under numbers 4 to 9 inclusive gave slightly more methyl red positive reactions with the 0.5 per cent Witte peptone broth incubated for 5

days than with the substituted 0.75 per cent Difco incubated for 2 days, while with those grouped under reference numbers 10 and 11 agreement between the original reactions in Difco and in the standard broth was complete. The number of anomalies discovered would probably have been even greater had we been able to use the standard medium throughout this work; but our purpose of segregating atypical forms was served in a measure by the provisional grouping based upon the reaction in Difco peptone broth.

The methyl red reaction was tested after 2 days' and after five days' incubation in standard Witte broth: but 1 strain (9c) was acid after two days and alkaline after five days; all the rest gave concordant results for the two incubation periods at that time.

Of the 25 uric acid positive strains, two (9a and 9b) are methyl red negative and have the general characters of *Bact. aerogenes.*

Ten strains give anomalous results with the methyl red and the Voges-Proskauer reactions: Nos. 4 and 5a fail to ferment salicin, dulcitol, adonitol, starch and glycerol. The last of the 9c strains and the first seven of the 10b strains are positive to both methyl red and the Voges-Proskauer reaction; and the last listed, 10b, is negative in both these reactions; two of these strains were lost before salicin tests were made; but the characters of these eight strains are predominantly those of the aerogenes type.

Thirteen strains are uric acid positive, methyl red positive with the Voges-Proskauer reaction negative or not recorded: 5b fails to ferment salicin, dulcitol and adonitol and can not be assigned a place; the two strains 6a resemble *Bact. coli* in most respects; 6b is sucrose, salicin, dulcitol positive, and is probably *Bact. aerogenes;* the first two 9c strains resemble *Bact. communior;* 10a is perhaps more like *Bact. aerogenes* than *Bact. communior;* 10c liquefies gelatine and ferments all carbohydrates, etc., save starch and dulcitol: the first four strains numbered 10d ferment actively all the carbohydrates, etc., except starch and glycerol; the last 10d fails to ferment salicin, starch and glycerol. Whether these 13 strains, departing more or less from type forms, are to be regarded as colon forms from soil or as

intermediate between the colon and aerogenes types, their positive uric acid reaction seems to mark them as without significance as indicators of pollution. There is a tendency of strains grouped under 9 and 10 to approach the maximum of fermentative power ascribed by Winslow and his co-workers to *Bact. aerogenes* as the most primitive type of the entire colon-typhoid group.

Five uric acid negative strains are acid to methyl red and Voges-Proskauer negative: the two 11c strains ferment all carbohydrates, etc., tested except starch: 11a resembles *Bact. communior* in that it is salicin and dulcitol negative; the two strains marked 11b approach *Bact. coli*.

The strains 11c and 10d were isolated from apparently unpolluted wells, respectively 1300 and 2000 feet deep.

The remaining strains are spore bearers, excepting No. 17, which lost most of its original characters under laboratory cultivation. They resemble the strains 4, 5a and 5b in their failure to ferment salicin, dulcitol and adonitol.

The relative value of the three criteria for discrimination of high and low ratio groups with these strains appears from the following considerations: 15 uric acid positive strains ferment mannitol, maltose, glucose, lactose, sucrose, salicin, dulcitol adonitol, and glycerol, or all save one of the last three and are considered as probable *Bact. aerogenes* forms; 2 are methyl red negative; 7 give the Voges-Proskauer reaction, and 1 is variable. There are 13 discrepancies in the methyl red and uric acid tests, and 7 or 8 in the uric acid and Voges-Proskauer tests. Two uric acid positive strains which failed only in fermenting dulcitol were not tested with salicin: 1 was acid to methyl red and was Voges-Proskauer positive; 1 was negative in both reactions. The uric acid test was confirmed by one of these reactions and negatived by the other in each instance.

Two strains (11b) fermented all the above listed sugars, etc., save glycerol, but were uric acid negative, methyl red positive, Voges-Proskauer negative in repeated tests. These are the only instances which raise a question as to the validity of the Uric acid reaction, which in all other cases cited appears preferable in discriminating high ratio members in waters.

DISCUSSION OF RESULTS

It is apparent that in our results, as in instances earlier cited, the methyl red and Voges-Proskauer reactions of the same strain are not always of opposite signs. Variation in each, but principally in the former, has been observed in the standard broth. Ayers and Rupp (1918) have shown that reversion of acid reaction exhibited by *Bact. aerogenes* cultures is due to the secondary decomposition of organic acids and is accompanied by rapid destruction of formic, acetic and other acids. With *Bact. coli* they noted that acid formation does not run parallel with the destruction of glucose, formic acid remaining constant or being slightly reduced during the later stages of fermentation. The distinction between *Bact. coli* and *Bact. aerogenes* is considered as lying chiefly in the difference in rate between the preliminary decomposition of sugars into acids and the secondary decomposition of the acids themselves. The suggestion was made by Burton and Rettger that there may be a difference in the rate of the secondary decomposition even in strains of *Bact. aerogenes* which would explain divergence in the atypical strains such as we are considering. They found the Voges-Proskauer reaction more reliable than the methyl red reaction, as cited above. The simpler uric acid reaction may prove even more dependable than the complex Voges-Proskauer reaction, being accomplished in briefer period and permitting less modification of the strain under cultivation. It is true that Rettger and Chen found it possible to shorten the incubation period from five days to twenty-four hours (even ten to fourteen hours) without altering the Voges-Proskauer reaction; they also report the successful use of Difco peptone in this test. But even with this reduction in the time element the possibility of variation in the complex reaction is not removed.

The positive uric acid reaction overlaps the acid methyl red and the negative Voges-Proskauer reactions in many strains. If the observation is confirmed, that the Koser reaction gives satisfactory correlation with the other reactions except among colon strains from soils, this may prove of value in clearing up

such anomalies as have been emphasized in this work and mentioned in the earlier cited references.

A considerable number of methyl red positive strains gave deferred fermentation of lactose (after three days) which is in line with the observations of Bronfenbrenner and Davis (1918) on *Bact. coli* from foods. Similar behavior of colon forms in gentian violet broth has been ascribed by Hall and Ellefsen (1919) to the inhibitive effect of the dye: it may have been in part attributable to the inhibitory effect of lactose itself, noted by Smith (1893), confirmed by Burling and Levine (1916), and recognized in the latest recommendation of the committee of the American Public Health Association (1920), which reduces the percentage of lactose in broth to 0.5 per cent. If our observation holds, that 0.2 per cent of lactose and other sugars and alcohols suffices in culture broths, some relief might be expected from delayed development of gas; but our strains reacted similarly in both 1 per cent and 0.2 per cent sugar broths throughout. In the most striking instances of erratic behavior studied there was no marked difference in the time of beginning gas production by typical and atypical forms.

The list of anomalous strains is undoubtedly incomplete: Levine, Burton and Rettger, and Burton in his thesis, to which we have had access through the courtesy of the author, Bronfenbrenner and Davis, and Rettger and Chen suggest abundant material not yet reducible to the fixed categories of any classification. This may be said also of the sporebearing forms; although some of them can hardly be confused with typical *Bact. coli*, several are likely to lead to misapprehension as to the safety of a water supply.

INTERPRETATION OF LABORATORY RESULTS

If the atypical strains, sporebearers and vegetative forms herein listed and cited, are considered as intermediate or transition forms from accepted colon or aerogenes types, the question of sanitary interpretation in unavoidable. Any of the lactose fermenting organisms (29 of the 31 especially studied) would lead to the condemnation of a water supply according to the

United States Treasury Department (1914) standard if more than two organisms were found in 100 cc.; of these 29, 3 are sporebearers, 1 is typical *Bact. aerogenes* not necessarily of fecal origin (Rogers 1918), 9 are anomalous with respect to those reactions accepted as indicative of high and low gas ratio; 16 are within the class of low ratio organisms on the basis of the methyl red and Voges-Proskauer reactions, and of these but 3 uric acid negative strains conform to recognized types.

An organism which requires prolonged invigoration to be restored to, or to acquire, conventional reactions with sugar broths and other media is far removed from the organism typical of fecal pollution. Considering the opportunity thus afforded for change in the original characters, a conclusion as to what must be regarded as essential indicators of pollution must take into account the undoubtedly wide variation of bacilli of the general colon-aerogenes group occurring in waters. Invigoration might lead an organism, long away from, or originating quite outside, the alimentary tract, to acquire the characters of typical fecal inhabitants. While it is important to ascertain the ultimate genetic relation between members of the group, it is one thing to say that these forms are of common, remote origin, and a very different one to say that the existent, feebly reacting, yet convertible forms are identical with, and of equal diagnostic importance with, organisms freshly isolated from feces under laboratory conditions: that is, to attribute to them as originally present in a water supply all the newly acquired characters. The uric acid reaction, however, admits of repetition without change so far as we have found with the limited number of strains isolated.

It is important that the laboratory procedure be as quickly completed as is reasonably possible, and that characters be neither lost nor acquired. Water seriously polluted shows gas production within much less than 24 hours. The readiness of strains to react is perhaps of greater diagnostic significance than the appearance of gas at twenty-four hours and at forty-eight hours as now observed. Levine (1920) considers the rate of gas production more significant than the total volume of gas formed. Burton suggested shortening preliminary enrichment

to avoid development of *Bact. cloacae*. The committee's recommendation (1912) for enrichment with transfer "as soon as gas is formed (usually in sixteen to twenty-four hours)" has persisted in many laboratories and is perhaps worth reviving officially, not so much to avoid overgrowth as to prevent undue modification. For the same reason the Voges-Proskauer reaction tested at the end of ten to fourteen hours' incubation in available American peptone broth is preferable to reliance upon the methyl red reaction, which requires 5-day incubation in a broth for which materials are not generally at hand. The uric acid test seems worthy of at least provisional acceptance because of the simplicity of the reaction and the facility afforded for confirming or correcting the somewhat erratic results observed in the Voges-Proskauer reactions of soil and water borne strains.

SUMMARY

Strains isolated from natural waters are grouped by their lactose, uric acid, adonitol, and methyl red reactions in Difco peptone broth (0.5 per cent and 0.75 per cent), and 35 strains especially studied are so arranged as to make evident the conflict between the Voges-Proskauer reaction and the methyl red reaction of strains in standard Witte peptone broth. There is lack of agreement in the discrimination of high and low ratio types. The uric acid positive reaction correlates best with the characters of the aerogenes type in carbohydrates, etc.

Upon the assumption that the uric acid reaction of colon forms from soils sufficiently characterizes them, this reaction may prove useful in checking and correcting the assignment of strains to the low ratio type indicative of possible fecal pollution.

Five sporebearers were isolated. It is probable that the number of these and of other anomalous forms is far less than would have been discovered had we been able to use Witte peptone in all methyl red tests.

The sugar reactions of members of the larger group seem to be as well tested in 0.2 per cent sugars as in the 1 per cent broths of the old standard procedure.

For the purpose of sanitary examination of waters it is desirable that the laboratory procedure be completed as early as con-

sistent with fairness to avoid change in characters, and to this end those methods are preferable which involve the simplest reactions and the briefest incubation periods.

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BOTULISM IN CATTLE

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The etiologic factor, or factors, in a sporadic toxemic-like disease in cattle sometimes designated as forage poisoning have been the subject of many experimental studies in the last decade. During this time the disease has occurred sporadically with varying severity throughout the middle western states, and more recently our attention has been repeatedly invited to these losses. It may be significant to mention that corn silage of some character was being fed to many of the herds developing the disease that came under our observation during the winter months of 1919–1920, yet this feed was definitely incriminated in but three instances. The primary relation of *B. botulinus*-like organisms to one type of forage poisoning in horses and mules, together with the occasional occurrence of this anaerobe in different animal feeds, has suggested the importance of determining the relation, if any, of certain toxic anaerobes to so-called forage poisoning in cattle, and our investigations of the disease in these animals have been devoted primarily to the pathogenic and toxic characters of spore bearing anaerobes in suspicious feeds, and of like organisms encountered in the intestinal content and spleen of animals fatally afflicted.

CLINICAL SYMPTOMS

In view of the fact that so-called forage poisoning in cattle may apparently be confused with hemorrhagic septicemia or enteric bacterial infections of the colon-typhoid group, or other rapidly fatal diseases of a toxemic character, a brief description

of the symptomatology of the disease under investigation is given. A differential diagnosis based upon clinical symptoms and gross anatomical findings at death may perplex the clinician and autopsist in many outbreaks. As noted to date in several affected animals, an acute and chronic symptom-complex of food or forage poisoning may be recognized in cattle. In the latter, weakness, local paresis, emaciation, muscular stiffness and decumbency are noted in varying degrees. Clonism developing without premonitory symptoms, terminating in sudden death, or followed by complete relaxation and recovery, marks the acute form of the disease. The nervous manifestations may be of a vicious character resulting in violence to feeding troughs, mangers or fences. Noticeable symptoms are not observed in the peracute disease preceding the agonal clonic spasm. Animals may remain decumbent for two or more days before death, during which time dyspnea, opisthotonus, coryza, lacrymation and catarrhal conjunctivitis often develop. One fatal spontaneous case delivered to our laboratory suffered from a secondary bronchial pneumonia, disclosed at autopsy. Several unsuccessful attempts to administer medicine by the mouth to the animal before death were probably associated with the development of the pneumonia, which was clearly of medicinal or mechanical origin.

In the more chronic cases animals may display visual disturbances. An estranged or frightened attitude on being approached, or a staring expression of the eyes, is noted. Emaciation and weakness contribute to an ill nourished cachectic appearance. Contraction of the flexor tendons in the posterior limbs, resulting in an extension of the metatarsalphalangeal articulation ("cock ankle"), with incoördination of movement, is not an uncommon complication, and animals may appear stiff, with a noticeable nervous attitude, and even loss of control in the anterior limbs, on being suddenly approached. Restraint or excitement of animals suffering from the chronic disease, accompanied by running or violent exertion, may terminate fatally from cardiac failure. In the acute type of bovine botulism partial or complete pharyngeal paresis is not uncommon,

yet in the chronic disease the appetite as well as organs of deglutition appear quite normal. The body temperature remains essentially unchanged, with slight fluctuations upward which are of short duration unless associated with secondary infection. Subnormal temperature and obstinate constipation are invariably present.

SUSCEPTIBILITY

Preliminary observations indicate that cattle between the ages of six months and two years are most susceptible, while older animals may also be affected. The mortality varies between 2 and 10 per cent though in extreme outbreaks a loss of 30 to 70 per cent or higher may occur. From observations it would appear that some cattle have, or acquire, an immunity to certain types of poisonous substances in feed, yet the natural resistance possessed by cattle of all ages to the type of intoxication under consideration is not always sufficient to protect against more or less serious constitutional disturbances. In more resistant animals death is not induced, yet the growth and development of the affected animal may be temporarily or permanently impaired. The mortality of the disease in many outbreaks may thus be secondary to the loss sustained by failure of the animals to increase in weight, by the decrease in milk flow in dairy cattle, or the loss of the feed in case the contaminated ration can be detected. Moreover it is believed that symptoms of *B. botulinus* intoxication in resistant cattle may thus be transitory and of an indefinite character, and that bovine forage poisoning may even prevail unrecognized as a distinct disease, manifested only by unthrift and malnutrition. In the light of recent observations the writers have probably failed to recognize the disease in cattle as a clinical entity in several outbreaks during the period of 1912–1917.

CAUSATIVE FACTOR RELATED TO RATIONS

In clinical outbreaks of the above character, bacteriological evidence has seldom been obtained to sustain or refute a presumptive diagnosis of a food or forage poisoning. As a matter

of fact the cause of the disease as it occurs throughout the Mississippi Valley has been satisfactorily established in but few outbreaks. Notwithstanding negative findings relative to the cause or causes involved, the recurrence of a clinical toxemic-like disease in cattle in the feed lots and pastures of Illinois and other middle western states lends evidence to the possibility of a distinct entity of forage poisoning, based upon our clinical conception of food poisoning in other domestic animals, i.e., in horses and mules (Graham, Brueckner and Pontius (1917)). In these animals the cause of death in several sporadic outbreaks has apparently been definitely associated with certain types of *B. botulinus* intoxication, as demonstrated by bacteriological findings in the feed and confirmed by the apparent protective value of specific antitoxin in susceptible animals receiving contaminated rations.

Feeding experiments (Rusk and Grindley (1918)) have been conducted by different investigators in an attempt to reproduce the disease in cattle. An accomplishment of this character would obviously afford an oportunity to inaugurate more definite and extended bacteriological studies, looking to the establishment of an etiologic factor. Experimental results in cattle feeding projects, together with the natural resistance of some animals accompanied by the abrupt or irregular termination of the spontaneous fatal disease in natural outbreaks, have in a broad sense failed to incriminate the rations specifically. However, in many outbreaks it appeared that the causative factor or factors were related to, if not incorporated in the feed. With this conception of the etiological relation of the feed to the disease, bacteriological studies have been extended to numerous samples of feed from suspicious outbreaks of this disease. The possibility of the disease or diseases encountered being associated with *Pasteurella boviseptica*, or toxic aerobes of the colon-typhoid group prompted animal inoculation and cultural methods to eliminate these microorganisms in tissue specimens.

Moulds have been mentioned in a more or less definite way in connection with forage poisoning in cattle and horses. A variety of these organisms have been encountered upon animal feeds

and it is suggested that these organisms may apparently be associated with the disease or may serve as causal agents in a secondary etiologic capacity, since experimental evidence in animals, to demonstrate the primary toxic character of certain organisms of this class per se, is unconvincing. If deductions are to be drawn at this time from a review of the literature and experimental evidence at hand in our studies, it appears that the moulds encountered are probably not of widespread primary importance in the toxemic-like disease of animals in question, as it occurs throughout the Mississippi Valley.

The writers have observed outbreaks of so-called forage poisoning in equines which were quite definitely related to the consumption of feed containing *B. botulinus* toxin. Susceptible animals (horses and mules) could be protected against the toxin in the feed by a prophylactic injection of botulinus antitoxin (Graham and Brueckner (1919)). The relation of *B. botulinus* of human origin (type B), was also noted by immunologic tests, while Burke of California has more recently incriminated *B. botulinus* (type A) in forage poisoning in horses (Burke, 1919). This strain has not been encountered to date in outbreaks of equine botulism coming under our observation.

RESISTANCE OF BOVINES TO BOTULINUS TOXIN

Following preliminary field observations of bovine forage poisoning in Illinois, Rusk and Grindley state

> The results of these investigations seem to indicate that most cattle are not so susceptible to forage poisoning as are horses and mules, and that contaminated corn silage, and possibly other animal feeds which are unsafe or fatal to horses, may be fed with less danger to cattle . . . . however, the evidence from many outbreaks leads the authors to suspect that some cattle are more susceptible than others and that damaged or otherwise contaminated corn silage, or possibly other feeds, may in some instances produce fatal results in cattle following ingestion.

Cattle have been fed rations spontaneously contaminated with botulinus toxin (type B) without manifest symptoms of illness other than loss of body weight, and mature cattle have con-

sumed ten to twenty lethal equine doses of botulinus toxin (type B) at one time in wholesome feed without inducing noticeable symptoms. In fact our observations indicate that a mature ruminant may possess marked resistance to botulinus toxin (type B) in the feed.

Information relative to *B. botulinus*-like organisms and their relation to forage poisoning in cattle, if any, has been eagerly sought in natural outbreaks, yet the degree of tolerance experimentally observed in mature, healthy experimental cattle to botulinus toxin (type B) suggested the possibility of an independent factor in this disease of bovines, and until recently the spontaneous occurrence of forage poisoning in cattle, wherein the rations proved to be contaminated with *B. botulinus*-like organisms, was in our observations without convincing bacteriological and immunological evidence.

SILAGE CONTAMINATED WITH B. BOTULINUS

In January, 1920, a sample of silage (Laboratory index 126), was received from Mr. L. W. Wise of Iroquois County, Illinois. It was stated that the sample in question was representative of feed which had apparently proven injurious to a herd of forty-seven pure bred cattle of all ages. Upon physical examination the silage did not show noticeable indications of spoilage. There were scant circumscribed colonies of wild yeast (*Monilia candida* Bon) on some of the leaves, which was identified in pure cultures by Professor H. W. Anderson, Assistant Professor of Pomology, University of Illinois. The colonies of yeast were visible only on close examination and the specimen could not be regarded as mouldy in the general sense that some feeds harbor organisms of this class. Indefinite evidence which pointed to the disease producing properties of the silage consisted of symptoms of illness observed in several (18) cattle, and as described by the owner, included inappetance, marked emaciation, constipation and general unthrift, with some transitory nervous manifestations (see fig. 1). Four animals chronically affected died. The younger animals of the herd were apparently not as susceptible as the mature full grown animals, or it may be pre-

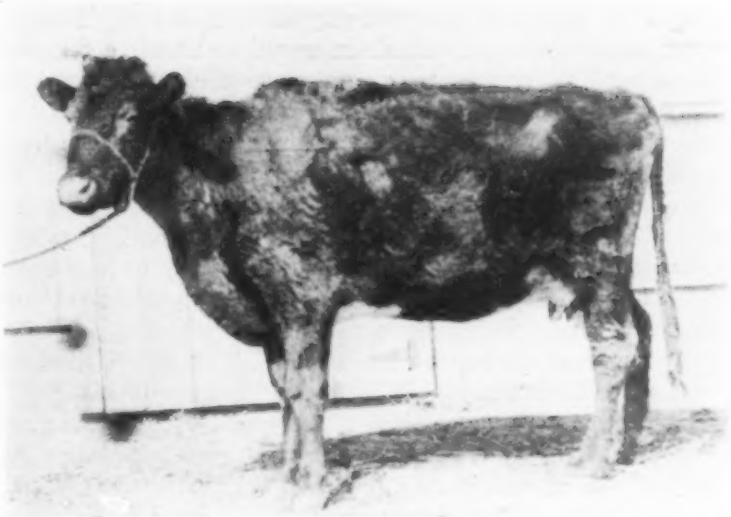

FIG. 1. A COW SUFFERING FROM A CHRONIC FORM OF BOTULISM AS A RESULT OF CONSUMING CONTAMINATED CORN SILAGE NO. 126

sumed that the older animals consumed more of the silage. The owner noted that trough space prevented the small animals from getting as liberal a portion of the feed as the older animals secured. Simultaneously with the marked illness and death of the animals, feeding of silage was discontinued and the herd improved. After an interval of three weeks the cattle were again allowed to eat of the silage in small quantities and illness again appeared in some animals of the herd. The symptoms were analagous to the manifestations noted in the original outbreak, but the silage was promptly eliminated from the daily ration and the affected animals improved and made a complete recovery. This experience suggested to the owner that the silage could not be safely used for feeding purposes, and opportunity to observe the effect of the continuous feeding of the silage in this herd or to other cattle or horses was not provided.

The clinical illness in these cattle on two separate occasions was at marked variance with experiments in feeding rations spontaneously contaminated with botulinus toxin to horses and mules, in that the character of the disease in cattle was chronic and slowly fatal. Furthermore, the manifest symptoms reported in this herd had not been noted in feeding *B. botulinus* contaminated silage to cattle, yet the anamnesis appeared somewhat in keeping with other spontaneous outbreaks of a disease of unknown etiology occurring in cattle throughout the corn belt states.

While clinical deductions might have suggested the presence of a poisonous substance in the silage, there appeared two important possibilities for consideration in this assumption. (a) The poisonous substance in the silage was not overcome by the natural resistance of the animals, or (b) the illness was induced by bacterial agents, chemicals, et cetera, unassociated with the silage and not mentioned by the owner. No feeding experiments were conducted to incriminate the silage further, but a bacteriologic examination of this feed was made.

BACTERIOLOGICAL FINDINGS

A sample of silage (50 grams) received for examination was immersed in sterile water and allowed to macerate in a dark place twenty-four hours at a temperature of 22°C. The sample

was then gently shaken and the liquid content removed to a sterile flask. Small particles of visible silage were removed by filtering through four layers of sterile gauze. The filtrate was then seeded in shake agar culture and heated fifteen minutes to 80°C. to destroy vegetative bacteria. The inoculated tubes were quickly placed in a cold water bath and allowed to solidify. On the surface of the agar to a depth of 2 to 6 cm., equal parts of agar and glycerol containing 1 per cent phenol were added to insure anaerobiosis. Ten days later the cultures, after incubating at 22°C., were examined and in one of the fifteen dilutions planted there was gas formation in the base of the tube, though distinct colonies were not visible. Anaerobes encountered in animal feeds, in our observations, are favored by the addition of glucose to the media, yet the numerous saprophytes encountered may outgrow and even disguise the presence of *B. botulinus*-like organisms. It is true that *B. botulinus* does not thrive on agar, yet it seems to develop slowly in plain agar shake cultures at 22°C. to 25°C. with limited gas production. Subculturing in glucose pork agar and transferring colonies to glucose pork broth (faintly alkaline) was employed to determine the toxic character of anaerobes cultivated in agar after ten days incubation in vacuum or hydrogen atmosphere. The normal toxicity of newly isolated *B. botulinus*-like organisms in broth cultures may not be characteristic or fully acquired until the second or third transfer at intervals of seven to ten days. The cultural characters and toxic quality of *B. botulinus* from silage as observed in guinea pigs, is illustrated in table 1; and in figures 2 and 3. All animals succumbed with the symptoms characteristic of *B. botulinus* intoxication.

TABLE 1

NUMBER	WEIGHT	DATE	TOXIN 126	RESULT
1	250	1/20/20	0.1 per os	Died 1/22/20
2	250	1/20/20	0.1 per os	Died 1/23/20
3	250	1/20/20	0.1 per os	Died 1/22/20
4	250	1/20/20	0.1 per os	Died 1/21/20
5	250	1/20/20	0.1 per os	Died 1/21/20
6	250	1/20/20	0.1 per os	Died 1/21/20
7	250	1/20/20	0.1 per os	Died 1/21/20

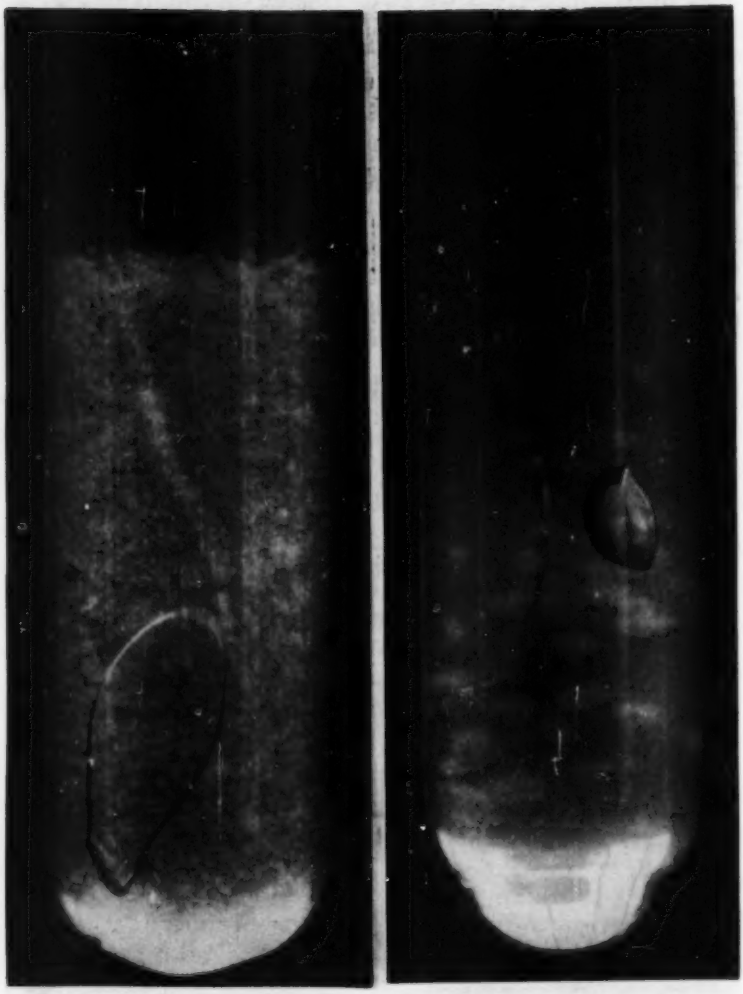

FIG. 2. GLUCOSE SHAKE AGAR CULTURES OF B. BOTULINUS ISOLATED FROM SILAGE NO. 126

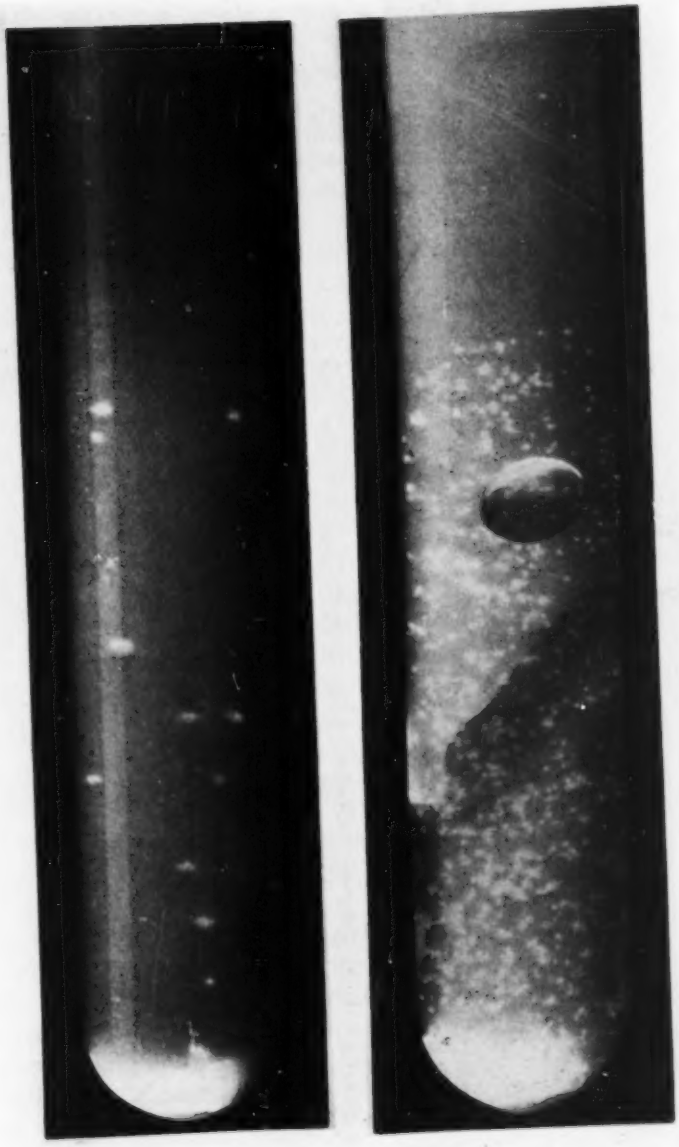

FIG. 3. GLUCOSE SHAKE AGAR CULTURES OF B. BOTULINUS ISOLATED FROM SILAGE NO. 126

IMMUNOLOGICAL FINDINGS

Immunological tests upon guinea-pigs using unfiltered broth cultures of the toxic anaerobe isolated from corn silage (126) and botulinus antitoxin prepared from a heterologous strain of *B. botulinus*, gave evidence of the identity of the toxin and according to Burke's classification (1919) proved to be of type B variety. The strain possesses the usual pathogenic characters for small laboratory animals and is culturally analagous to

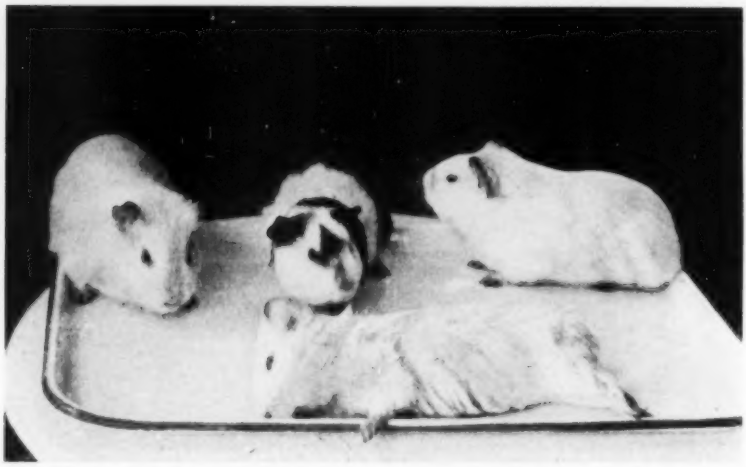

FIG. 4. IMMUNOLOGIC TEST SHOWING RELATION OF STRAIN OF TOXIN FROM SILAGE NO. 126, B. BOTULINUS ANTITOXIN (TYPE B)

The three pigs in the rear received the serum and toxin. The control pig received the toxin only.

other strains of *B. botulinus*. Botulinus antitoxin (type B) proved efficacious in small animals against many lethal doses (c. f. 100) of toxin per os. An arbitrary toxic unit of 0.001 cc., which represents the minimum lethal dose, when given per os to guinea pigs of a given weight, has been tentatively used in determining the relative potency of antitoxic serum. This toxic unit per os in guinea-pigs weighing 250 grams may produce symptoms in twenty-four to forty-eight hours and is invariably

followed by death on the ninth or tenth day. Results of immunologic tests in guinea-pigs as in table 2 illustrate the specific relation of the *B. botulinus* strain from the corn silage sample 126 to type B variety (see fig. 4). One to two cubic centimeters of antitoxic serum of the desired potency has repeatedly proven efficacious against 100 minimum lethal toxic units given separately by the mouth at the time or a few hours after the anti-

TABLE 2

NUMBER	WEIGHT	DATE	SERUM TYPE B	TOXIN 126	RESULT
1	780	1/21/20	2 cc. subcutaneously	0.1 per os	Healthy
2	785	1/21/20	2 cc. subcutaneously	0.1 per os	Healthy
3	800	1/21/20	2 cc. subcutaneously	0.1 per os	Healthy
4	775	1/21/20	0	0.1 per os	Died 1/23/20

serum, while 0.001 cc. of antitoxin of sufficient strength per gram weight suffices to protect a guinea-pig against 100 minimum lethal doses of toxin given by the mouth. Guinea-pigs varying in weight from 200 to 800 grams, owing to shortage of pigs of uniform weight, have been employed to note the specific relations of the toxin to the antitoxin of types A and B. In table 2 the relation of strain 126 to type B immune serum is tabulated.

SERUM TREATMENT OF CATTLE

The preliminary bacteriologic and immunologic studies herein enumerated suggested that the losses in cattle consuming the silage was probably associated with *B. botulinus* intoxication. This conclusion was practically established in the laboratory when it was learned that several tons of the silage in question were to be condemned and discarded as unfit for feeding purposes. In view of the preliminary findings the advisability of recommending that this silage be fed to the cattle seemed logical to us, providing the animals in the herd might first be injected with botulinus antitoxin. It was believed that the practical value of specific antitoxic serum in cattle for the prevention of botulism might be observed and possibly definitely demon-

strated under natural conditions comparable to field tests with horses (Rusk and Grindley) wherein the value of antitoxin was apparently observed.

Dr. I. B. Boughton of the Animal Pathology Division, University of Illinois, with the consent of the owner treated 43 cattle of the original herd with antitoxin, type B. Amounts varying from 30 to 50 cc. were injected subcutaneously into each animal. A control or untreated animal was placed in the herd with the 43 treated cattle. Following the injection of serum the silage which had previously proven injurious to cattle, and which upon examination proved to be contaminated with *B. botulinus* (type B) was fed in liberal amounts for sixty consecutive days, until the supply of silage was exhausted. No symptoms of illness were noted in the treated animals and the one untreated animal.

The protective value of type B serum in these animals must be discounted, in the opinion of the writers, for the reason that a degree of immunity might have been developed by a previous illness which had occurred in approximately one-half of the animals of this herd and which in all probability was induced by botulinus toxin in the silage. The control animal did not succumb or even display clinical symptoms of illness, and therefore no precise and definite deductions can be drawn, yet the protective value of botulinus antitoxin in laboratory tests suggests the possible value of this antitoxin in combating *B. botulinus* intoxication in cattle, as well as the advisability of further tests of this character in the control of natural outbreaks of this disease in bovines.[1]

[1] As this manuscript is being prepared the importance of a polyvalent serum in further trials is suggested by the results of bacteriologic and immunologic findings in two separate and distinct outbreaks of botulism in cattle occurring near Paxton, Illinois, wherein A and B types of B. botulinus respectively were encountered.

During the feeding test, the owner advised that the silage in question had been fed independently to an untreated cow not included in the experimental group, with the result that the animal developed symptoms indistinguishable from the illness originally observed in the herd. This animal had not previously received the silage and the owner's observations seem worthy of record.

SUMMARY

1. An anaerobic bacillus biologically resembling *B. botulinus* (type B) was isolated from a corn silage (126).

2. Several (18) cattle of the herd consuming the silage in question developed symptoms of forage poisoning on two different occasions and four animals died. It is possible that botulinus toxin in the ensilage was primarily related to the disease in question.

3. The silage was regarded as unsafe for cattle and after discontinuing its use in the daily rations, the animals remained healthy.

4. Botulinus antitoxin (type B) proved efficacious in protecting guinea-pigs against lethal doses of toxin in unfiltered broth cultures produced by the anaerobic bacillus isolated from the corn silage (126).

5. An opportunity was afforded to inject forty-three cattle on this farm with botulinus antitoxin, and subsequently to feed them with the silage. The animals remained apparently healthy. One control or untreated animal did not show visible illness and the value of the antitoxin in the feeding operations is therefore not conclusive. It is worthy of record that the treatment did not injure the animals and encouragement is offered for more extensive field trials in determining the value of the antitoxin in cattle against the ill effects of otherwise nourishing rations containing *B. botulinus* toxin which heretofore has advisedly been discarded. The latter item is of importance considering the increased cost of producing grain and forage.

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NOTE ON THE INDOL TEST IN TRYPTOPHANE SOLUTION

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The application of the indol test to tryptophane solutions by H. Zipfel was, without doubt a great advance. The theoretical basis of this reaction is so generally[1] known that it is superfluous to give an explanation here.

In applying the method of Zipfel it has happened on different occasions, that I have failed to obtain growth (turbidity) in the tryptophane solution even with bacteria, which are known as strong indol liberators,[2] as for example *Bact. vulgare*. Of course in this case there is also no indol reaction with the reagent of Ehrlich (p-dimethylamidobenzaldehyde). It occured to me that this failure might be due to the hydrogen ion concentration in the solution in question.

Zipfel says nothing about the neutralization of the solution in either of his two publications on this subject and so far as I know, this fact has never been pointed out by any other worker. In an electrical determination of the hydrogen ion concentration, which I undertook on a tryptophane solution of the composition prescribed by Zipfel[3] I found the value of the pH = 5.41.

[1] Centralbl. für Bakteriol., Abt. I., Orig., **64**, 1912, 65; Centralbl. für Bakteriol. Abt. I., Orig., **67**, 1913, 572.

[2] The term "liberation" is better than "formation," as the action is merely a splitting up of the tryptophane molecule, with liberation of the indol group.

[3]

	per cent
Asparagin	0.5
Ammonium lactate	0.5
Potassium diphosphate	0.2
Magnesium sulfate	0.02
Tryptophane	0.03

As is obvious, this is a manifestly acid solution. The possibility that here was the explanation of the failure to obtain growth with *Bact. vulgare* now seemed very probable to me. An investigation of this question was therefore undertaken, which I wish to present in this paper. I have also attempted to answer another question, namely, whether or not it is necessary to have both asparagine and ammonium lactate present in the solution.

The researches, after a series of orientation experiments, which need not to be given here, were carried out in the following manner: 500 cc. of the tryptophane solution, but without

TABLE 1

COMPOSITION OF THE SOLUTION	pH	
	Before sterilization	After sterilization
With ammonium lactate, neutral	6.81	6.29
With ammonium lactate, neutral, Jena glass		5.97
With ammonium lactate, acid	5.21	4.83
With ammonium lactate, acid, Jena glass		4.69
Without ammonium lactate, neutral	6.81	6.23
Without ammonium lactate, neutral, Jena glass		6.03
Without ammonium lactate, acid	4.86	4.83
Without ammonium lactate, acid, Jena glass		4.67

ammonium lactate, was divided into two portions of 250 cc. each. To one of these was added 1.25 grams (0.5 per cent) ammonium lactate. Each one of these solutions was divided into two parts, of which one was left as it was, while the other was neutralized with N NaOH to litmus. All of these four solutions were then transferred to test tubes (10 cc. per tube). From each of these four solutions, Jena glass tubes were also made up for comparison. The pH was determined in all the solutions before and after sterilization, which was carried out in the autoclave at 118°C. momentarily. The results of these series are shown in table 1.

From the table it is seen that the solution itself is very acid, where it is not neutralized, and almost without exception the

hydrogen ion concentration increases during the sterilization. Furthermore, it follows from this that some alkali has been leached from the glass of the ordinary tubes, because the values of the pH of the Jena glass tubes are, consistently, lower (from 0.14 to 0.32) than for the others.

With these four sterilized solutions, indol tests were carried out with eight different species of bacteria, of which three are known to be strong indol liberators, namely *Bact. coli*, *Bact. vulgare* and *Vibrio cholerae*.[4] The other five are not indole liberators. These species were *Bact. aerogenes*, a variety of *Bact. Zopfii*, isolated from soil of the northern coast of Greenland, a motile non-sporeforming rod, isolated from the faeces of a crow and finally a yellow, non-motile non-sporeforming short rod isolated from the faeces of the musk ox. The two last were also obtained from Northern Greenland.

Of all these strains, one platinum loop from a twenty-four hour broth culture was inoculated in each of the above mentioned solutions. After incubation for twenty-four hours at 37°C., they were examined for growth (turbidity), as well as for the setting free of indol, by adding 5 cc. of the p-dimethylamidobenzaldehyde. According to Zipfel's work, which I can confirm in this point, it is quite unnecessary to let the tryptophane cultures stand longer than twenty-four hours at 37°C. If there is no growth in this time, it is of no use to continue the observation.

The results of these series are given in table 2.

If we consider at first only the influence of the hydrogen ion concentration, we find our suspicions confirmed that the non-neutralized solution is too acid always to permit the growth of the organism which is to be examined for indol liberation. *Bact. vulgare* and *Vibrio cholerae* do not grow and therefore naturally cannot give the indol reaction in the solutions which are not neutralized.

If we consider the results from the solutions with and without ammonium lactate, we may conclude from these experiments

[4] I wish to thank Prof. C. Kling, director of Statens Bakteriologiska Laboratorium, for his kindness in supplying me with the cultures of *V. cholerae* and *Bact. typhi*.

that it is of no consequence whether this compound is present or not. To be sure, in some cases the growth (turbidity) was stronger in the tubes which also contain ammonium lactate, but examples to the contrary are also to be noted, and in no

TABLE 2

CULTURE	+ AMMONIUM LACTATE NEUTRAL pH = 6.29	+ AMMONIUM LACTATE ACID pH = 4.83	WITHOUT AMMONIUM LACTATE NEUTRAL pH = 6.23	WITHOUT AMMONIUM LACTATE ACID pH = 4.83
Bact. coli:				
Growth	+++	−	++	+
Indole reaction	+	−	+	+
Bact. vulgare:				
Growth	++	−	(+)	−
Indole reaction	+	−	+	−
V. cholerae:				
Growth	+	−	++	−
Indole reaction	+	−	+	−
Bact. typhi:				
Growth	+	+	+	+
Indole reaction	−	−	−	−
Bact. aerogenes:				
Growth	+++	++	++	++
Indole reaction	−	−	−	−
Bact. Zopfii:				
Growth	++	−	++	+
Indole reaction	−	−	−	−
Bacteria from crow:				
Growth	(+)	−	(+)	−
Indole reaction	−	−	−	−
Bacteria from musk ox:				
Growth	−	−	+	−
Indole reaction	−	−	−	−

cases has the indol reaction given different results in the tubes with and without ammonium lactate.

The results of the experiments here related are that the solution of Zipfel is equally satisfactory even without ammonium lactate, but that under all conditions it must be neutralized.[5]

[5] It may happen occasionally that the tryptophane solution gives satisfactory results without neutralization, but this probably generally depends upon an especially strong leaching of the alkali from the glass during the sterilisation.

THE NATURE OF HEMOLYSINS

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Attention was first called to the fact that some bacteria produce hemolysins when Ehrlich (1898) showed that the bacillus of tetanus produced a substance which he called tetanolysin. The discovery of the existence of this lysin was rapidly followed by the announcement of other bacterial hemolysins, such as pyocyanolysin (Bulloch and Hunter, 1900; Weingeroff, 1901), staphylolysin by Neisser and Wechsburg (1901), streptolysin by Besredka (1901), typholysin by E. and P. Levy (1901), megatheriolysin by Todd (1901), etc.

It was soon shown that these lysins were characteristic of the organisms that produced them. For instance the staphylolysin, according to Neisser and Wechsburg, is injured by heating to 48°C. for twenty minutes, and destroyed at 56°C. for twenty minutes. Pyocyanin is destroyed by heating to 100°C. for fifteen minutes if it is free in the filtrate, but if the organisms are present it requires a higher temperature, and typholysin is not destroyed by boiling. Streptolysin requires 70°C. for two hours.

The majority of the lysins give rise to antilysins which are specific, though streptolysin is an exception. In fact the ability of a lysin to call forth an antilysin seems to run parallel with the ability of the microörganism producing the lysin to call forth antibodies to itself.

Lubenau (1901) considered the possibility that some substances which are known to be present in the medium may at times be responsible for the hemolysis. He tested the hemolytic power of sodium carbonate, ammonia, glucose and lactic acid, and showed that the strengths of these substances required to hemolyze are rarely ever present at the time the hemolysin is

active. Bulloch and Hunter showed that while a culture of *Ps. pyocyanea* is highly alkaline yet when the pH is brought back to near the neutral point it is still hemolytic though less so. This statement has been denied by Jordan who maintains that the hemolysis in this case is due to alkali.

In view of the light that has been thrown by Warden's work upon the composition of organisms, particularly in respect to their fatty complexes, it seemed logical to us that these fat antigens should be investigated as to the possibility of their playing a part in hemolysin production. This idea seemed particularly attractive because those organisms such as *Streptococcus* and *B. megatherium* which produce hemolysin early in their growth, and which also yield the most powerful hemolysins, are Gram positive, and the Gram positiveness of an organism is known to depend upon the presence of unsaturated fats. It is also well known that the unsaturated fatty acids and their salts are much better hemolytic agents than the non-volatile, saturated acids. We are aware, also, of the fact that if the fatty acid complexes should play a part in hemolysis the action would not be that following their simple suspension in salt solution because of the factors of a colloid nature introduced by the broth menstruum.

With these ideas in mind we decided to see if it were possible to produce an artificial hemolysin, using the fat complexes which were characteristic of the organism whose lysin we were trying to imitate. In order to do this ideally we realized that we must copy as closely as possible the condition existing in the medium at the time the hemolysin is at its height. The hemolysin first studied was that of the Streptococcus. The medium used throughout this work, called the standard medium, consisted of a veal infusion broth containing 2 per cent bactopepton and 0.5 per cent NaCl. The pH was varied from 7.1 to 7.9. In growing the Streptococcus organisms 10 per cent rabbit serum was added before inoculation. The cells used in the hemolytic experiments were fresh rabbit cells washed four times with 0.85 per cent salt solution, and made up in a 2 per cent salt solution suspension.

Figure 1 is typical of a number of curves derived when two different kinds of media were used, each having an original pH of 7.2, one being the standard medium, the other the standard plus 0.2 per cent glucose. All tubes were heated to 37°C. before

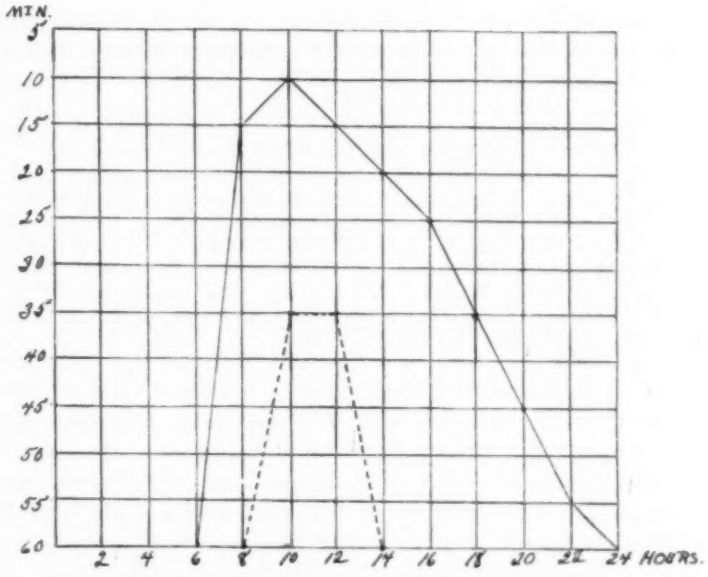

FIG. 1. SOLID LINE, PLAIN BROTH STANDARD MEDIUM
BROKEN LINE, GLUCOSE BROTH STANDARD MEDIUM

inoculating, and then inoculated with a 4 mm. loopful from a twelve hour 10 per cent serum-broth culture of *Streptococcus hemolyticus*. The tubes were incubated at 37°C. and every two hours one tube of each kind of medium was removed, a part being centrifugated for one-half hour at 1800 revolutions per minute, and the pH of the remaining portion determined. Then 0.1 cc. of the supernatant fluid was added to 1 cc. of cell suspension and placed in a water bath at 37°C. for one hour. The abscissae of the chart represent minutes required for complete hemolysis, the ordinates showing the age of the culture.

The pH of the standard medium was found to change to 7.0 at eight hours returning to about the original reaction at from eighteen to twenty hours. The pH of the medium containing glucose rose to 5.58 at twelve hours and returned to 6.10 at twenty hours.

The striking feature in the chart is seen to be the disadvantage of even small amounts of glucose for the production of hemol-

TABLE 1

A. Broth containing 40 mgm. per liter of K salts antigen.
B. Broth containing 40 mgm. per liter of Na salts antigen
C. Broth containing 40 mgm. per liter of fatty acid antigen

1 cc. of A plus 1 cc. of 2 per cent rabbit cell suspension.....	++ 20 minutes
1 cc. of B plus 1 cc. of 2 per cent rabbit cell suspension.....	++ 20 minutes
1 cc. of C plus 1 cc. of 2 per cent rabbit cell suspension.....	++ 25 minutes

++ indicates complete hemolysis.
– indicates no hemolysis.
The above mixtures remained perfectly clear. They were completely inactivated upon heating at 65°C. for thirty minutes.

ysin. Not only was the hemolysin weaker but of much shorter duration, though the specimens were centrifugated in the same centrifuge for the same length of time.

We also determined the strength and duration of hemolysin, starting with a pH of 7.8 in the standard broth, but found no striking difference from that of the 7.2. In growing these cultures and in testing the strength of the hemolysin and the time in which it appeared, two points were impressed upon us, first, that noted by other workers, that the hemolysin occurs earlier and is much stronger if the culture from which the transplant is taken is young, preferably not over twelve hours old, second, that using 0.1 cc. of culture for the transplant instead of a loopful caused the hemolysin to appear earlier in the incubation.

In attempting to produce an artificial hemolysin the standard medium was used. We omitted the serum because it was found that it distinctly interfered with hemolysin production, just as it also interfered with the lytic power of natural hemolysin if added after the lysin appears. We believe that the function of the serum in growing Streptococcus is to insure rapid and abundant growth, which is apparently essential for the production of

lysin, and that the colloidal property of the serum which interferes with the action of the streptolysin is undoubtedly destroyed by the growth of the organism. We shall show further on that serum added to a medium in which an organism (*B. megatherium*) produces strong lysin without it, interferes markedly with lysin production.

The antigen used was the fat complex, in the form of the fatty acids and their salts, found by Warden to be characteristic for the Streptococcus. The sodium and the potassium salts of the complex were made up in alcoholic solutions of such strengths that 1 cc. contained respectively 10 mgm. and the solution of the fatty acids such that 1 cc. contained 20 mgm. and consequently the amounts of alcohol necessary to add to secure the concentration of antigen desired was never sufficient to cause a change in the appearance of the broth or to have any hemolyzing effect on the red cells. The antigens were added by means of 1 cc. pipettes graduated in hundredths and thoroughly mixed with the broth, taking care to avoid foam. The amounts used varied between 32 mgm. and 120 mgm. per liter, these quantities apparently having no appreciable effect on the pH. We noted in some of the broth that clouding appeared after about 45 mgm. per liter had been added, whereas other broths remained clear with 60 mgm. per liter. One factor in this regard appeared to be the color of the broth—the darker the color the more antigen it would take up without clouding. Another important observation was the variation in the amount of antigen per liter required to make the broth hemolytic, in some instances 30 mgm. sufficing, in others 50 mgm. These differences were found to be due to slight variations in the manner of emulsifying and in the time the mixtures were allowed to stand. Table 1 is a specimen protocol of the hemolytic power of the artificial emulsions.

Table 2 gives an example of the effect of pH upon the hemolyzing power and the temperature of inactivation of artificial emulsions made with the K salt and fatty acid antigens.

We realized that the broth containing the natural hemolysins must be quite different from the artificial emulsions we were

working with because of the action (digestive and otherwise) upon it of the microorganisms. What probably is more important is that it contained emulsifying substances which were

TABLE 2

A. pH of broth 7.1 with 60 mgm. K salt antigen per liter
B. pH of broth 7.1 with 60 mgm. acid antigen per liter
C. pH of broth 7.4 with 60 mgm. K salt antigen per liter
D. pH of broth 7.4 with 60 mgm. acid antigen per liter
E. pH of broth 7.9 with 60 mgm. K salt antigen per liter
F. pH of broth 7.9 with 60 mgm. acid antigen per liter

	AT ONCE	ONE HOUR	TWO HOURS	AFTER 65°C FOR THIRTY MINUTES
1 cc. of A plus 1 cc. rabbit cell emulsion.....	++12′	++9′	++9′	++23′
1 cc. of B plus 1 cc. rabbit cell emulsion.....	++17′	++15′	++12′	++28′
1 cc. of C plus 1 cc. rabbit cell emulsion.....	++11′	++10′	++12′	−1 hr.
1 cc. of D plus 1 cc. rabbit cell emulsion.....	++26′	++15′	++15′	−1 hr.
1 cc. of E plus 1 cc. rabbit cell emulsion.....	++19′	++12′	++15′	++40′
1 cc. of F plus 1 cc. rabbit cell emulsion.....	++30′	++16′	++14′	++45′

From the above experiment it will be seen that the artificial hemolysin is inactivated at 65°C. for thirty minutes at a pH of 7.4, but at concentrations of 7.1 and 7.9 the inactivation is only partial.

delivered into it with the disintegration of the bacteria. With this idea in mind we tried the effects of some emulsifying substances to see if we could imitate the natural process more closely, and to determine whether the broth would not hold more of the fats without clouding. The substances chosen were such proteins as hemoglobin, casein, and typhoid protein, with which the fat antigen was emulsified,—imitating the conditions we believe to exist in the germ bodies—prior to adding to the broth. The following table is an example of the results.

TABLE 3

A. 35 cc. of broth to which was added slowly in fractions 5 cc. of a solution containing 10 mgm. of typhoid protein and 2.4 mgm. of K salt antigen.
B. 35 cc. of broth to which was added in the same manner 5 cc. of a solution of 10 mgm. of casein with 2.4 mgm. of K salt antigen.
C. 35 cc. of broth to which was added in the same manner 5 cc. of a solution of hemoglobin with 2.4 mgm. of K salt antigen.

The proteins were dissolved in 5 cc. of salt solution and the solution of antigen mixed drop by drop with continual gentle shaking.

1 cc. of A plus 1 cc. of cell suspension..... ++ in 20 minutes
1 cc. of B plus 1 cc. of cell suspension..... ++ in 10 minutes
1 cc. of C plus 1 cc. of cell suspension..... ++ in 10 minutes

Control emulsions of like amounts of solutions of broth with the proteins alone showed no hemolytic power.

All the above mixtures remained perfectly clear, and were inactivated at 65°C. for thirty minutes. Other combinations were tried with amounts of fat antigen varying from 40 mgm. to 120 mgm. per liter, and with quantities of protein varying from 5 mgm. to 20 mgm. per 40 cc. These mixtures also remained perfectly clear.

The influence of the colloidal nature of the broth on these artificial hemolysins was so apparent that we were desirous of seeing whether alterations in the broth would affect any particular changes. To this end the ordinary standard broth was passed through a Berkefeld filter before emulsification with the antigens. A control unfiltered broth of pH 7.1 containing 32 mgm. per liter of K salt antigen in amounts of 1 cc. produced total hemolysis of 1 cc. of cell suspension in sixty minutes, whereas the filtered broth containing the same amount of the antigen gave no hemolysis whatever. By doubling the amount of antigen added to the filtered broth the hemolysis appeared and was complete in one hour. This experiment, repeated with the Na salt and with the fatty acid antigen, gave similar results, and appeared to indicate that filtration removed from the broth particles of some material instrumental in hemolysin production.

After it had been found that inactivation of the *B. megatherium* lysin could be brought about by various adsorbents, to be mentioned later, we attempted the same procedure with both the natural and artificial streptolysin. The results appear in table 4.

The results of inactivation by means of the surface of defatted colon bodies were the same as from starch. Also inactivation by the same adsorbents in the ice box over night instead of at 45°C. gave identical results. In short, the artificial antigen was readily inactivated by these methods but the natural lysin was not. We then tested out the inactivation of the artificial lysin when produced in 10 per cent serum broth. As stated pre-

viously it is difficult to produce artificial lysin in the presence of serum, but it is merely a question, of adding larger amounts of antigen to serum-broth than are required to render standard

TABLE 4

A. Twelve hour centrifugated Streptococcus culture
B. Artificial lysin with 60 mgm. K salts Strep. antigen
C. Artificial lysin with 60 mgm. Na salts Strep. antigen
D. Artificial lysin with 60 mgm. acids Strep. antigen

1 cc. of A plus 1 cc. cell suspension	++ in 10 minutes
1 cc. of B plus 1 cc. cell suspension	++ in 12 minutes
1 cc. of C plus 1 cc. cell suspension	++ in 14 minutes
1 cc. of D plus 1 cc. cell suspension	++ in 15 minutes

To 5 cc. of A, B, C, and D there was added a definite quantity of starch suspension and the tubes were placed in the water bath at 45°C. for 1 hour, together with control tubes without the starch suspension. After centrifugation of the starch the lysins were tested as follows:

A1, B1, C1, D1, represent the lysins treated with starch
A2, B2, C2, D2, represent the lysins untreated

1 cc. of A1 plus 1 cc. cell suspension	++ 40 minutes
1 cc. of B1 plus 1 cc. cell suspension	−
1 cc. of C1 plus 1 cc. cell suspension	−
1 cc. of D1 plus 1 cc. cell suspension	−
1 cc. of A2 plus 1 cc. cell suspension	++ 18 minutes
1 cc. of B2 plus 1 cc. cell suspension	++ 20 minutes
1 cc. of C2 plus 1 cc. cell suspension	++ 20 minutes
1 cc. of D2 plus 1 cc. cell suspension	++ 20 minutes

broth hemolytic. Much larger quantities of antigen can be added to serum-broth, if added in small amounts at a time, without clouding, than to standard broth. As a result of this experiment it was shown that with a Streptococcus serum-broth culture fourteen hours old, centrifugated, 1 cc. of which hemolyzed 1 cc. of cell suspension in twenty minutes and with an artificial K salts hemolysin containing 300 mg. antigen per liter, 1 cc. of which hemolyzed 1 cc. of cell suspension in five minutes attempted inactivation with the adsorbing substances in the ice box and at 45°C. produced no such effect, i.e., neither natural or artificial hemolysin was inactivated. This seemed to justify the conclusion that the reason we were unable to inactivate the

natural streptolysin was because of the presence of the serum and not because of any peculiarity of the lysin itself.

Several attempts were made to produce antilysin by injections into animals of the natural streptolysin, the artificial lysin and streptococci themselves, but the results were unsatisfactory. This work is being continued and it is hoped we may report on it later. This difficulty has been commonly encountered by other workers (Besredka, 1903).

The second microörganism selected for observation was *B. megatherium*. We chose this bacterium because from the work of Todd (1901) it was known to produce strong hemolysin in a comparatively simple medium, and give rise to good antilysin. The hemolysin of this organism is also very stable which is an advantage over streptolysin. The standard medium was used in all the experiments. We found that the lysin appeared as early as the tenth hour and lasted for weeks. The pH of the cultures, determined at two hour intervals was found to change to 7.2 or 7.3 when the original pH of the medium was 7.8. The addition of 10 per cent of rabbit serum to the standard medium before inoculation gave very much weaker hemolysin than when the standard broth was used alone.

The antigen used for the production of the artificial hemolysin was the fat complex found by Warden, Connell and Holly to be characteristic for the *B. megatherium*. The various solutions of the antigen were made in the same manner as those used in the work on the streptococcus, and the emulsification of the antigen with the broth was carried out similarly. Tests showed that artificial hemolysin containing 40 mgm. K salt antigen per liter gave complete hemolysis, in 1 cc. doses, of 1 cc. of red cell suspension in thirty minutes, that of Na. salt antigen of equal strength gave complete hemolysis in the same length of time; and that containing the acid antigen produced, in the same dose, complete hemolysis in twenty-five minutes. These artificial lysins were inactivated at 65°C. for thirty minutes.

The following table is an example of the effect of pH upon the hemolytic power, and upon the inactivation of the K salt and acid antigen of the *megatherium*.

TABLE 5

A. Broth pH 7.1 plus 60 mgm. K salts antigen per liter
B. Broth pH 7.4 plus 60 mgm. K salts antigen per liter
C. Broth pH 7.9 plus 60 mgm. K salts antigen per liter
D. Broth pH 7.1 plus 60 mgm. acids antigen per liter
E. Broth pH 7.4 plus 60 mgm. acids antigen per liter
F. Broth pH 7.9 plus 60 mgm. acids antigen per liter

	AT ONCE	ONE HOUR	TWO HOURS	INACTIVATION AFTER ONE HOUR AT 65°C
1 cc. of A plus 1 cc. cell suspension	++28′	++17′	++17′	++50′
1 cc. of B plus 1 cc. cell suspension	++35′	++19′	++20′	−1 hr.
1 cc. of C plus 1 cc. cell suspension	++36′	++25′	++25′	−1 hr.
1 cc. of D plus 1 cc. cell suspension	++20′	++13′	++15′	++38′
1 cc. of E plus 1 cc. cell suspension	++30′	++13′	++16′	−1 hr.
1 cc. of F plus 1 cc. cell suspension	++30′	++18′	++19′	−1 hr.

These emulsions remained perfectly clear.

The question of partial digestion of the broth on the part of the microörganisms during growth led us to attempt a similar procedure in the effort to copy as closely as might be the germ action in the production of our artificial lysin. We added a small quantity of pancreatin powder to the broth containing the antigen and digested the mixtures in the water bath at 45°C. for one hour. The result of this experiment was complete inactivation of the lysin, rather than one favorable to lysin production. The question then arose as to whether the loss of hemolytic power was not due to simple adsorption of the antigen rather than to digestion, and the following experiment showed such to be the case.

One cubic centimeter of artificial *B. megatherium* hemolysin containing 100 mgm. of the K salts per liter hemolyzed 1 cc. of cell suspension in ten minutes. Five cubic centimeter quantities of this hemolysin were treated with a definite amount of starch, and the same quantities with defatted colon bodies. These mixtures, together with 5 cc. controls of untreated hemolysin were placed in the water bath at 45°C. for one hour, and identical specimens were placed in the ice box over night. After

centrifugation at 1800 revolutions per minute to remove the adsorbent the fluids were tested for hemolytic power. Those that had been treated with adsorbent were completely inactive while the controls hemolyzed promptly in fifteen and in ten minutes.

We then tested the natural hemolysin of the *B. megatherium* to see if it could also be inactivated in the same manner. The culture used was an eighteen hour standard broth growth centrifugated at 1800 revolutions per minute and the clear supernatant fluid pipetted off. 0.25 cc. of this hemolysin hemolyzed 1 cc. of cell suspension in twenty minutes. The procedure with the previous adsorbents was then repeated with this natural lysin, with the result that the treated portions were found to be completely inactivated while the untreated controls gave complete hemolysis in fifteen minutes. In other words we found it possible to inactivate the artificial and the natural lysin by adsorption upon surfaces. Inactivation in this manner can be rapidly effected by heating to 45°C. for one hour, a temperature at which ordinary organisms do not grow, or more slowly by allowing the mixtures to stand in the ice box over night. Apparently the pancreatin powder acts, not by breaking up the fats but rather by simple adsorption. Attention has been previously called by others to the fact that pepsin and trypsin destroy the lysin of *Ps. pyocyanea*.

It was also found that previous emulsification of the *B. megatherium* antigen mixtures with hemoglobin, casein and typhoid protein before their addition to the broth gave results quite in accord with those obtained with the Streptococcus antigen.

Todd showed, as mentioned previously, that the lysin produced by *B. megatherium* when injected into animals gave rise to antilysin. We injected several groups of rabbits, some with natural lysin, others with the artificial hemolysin. These animals were given six injections subcutaneously, three at daily intervals and then, after an interval of four days, three more at daily intervals. On the seventh day after the last injection the animals were bled from the heart and the serums allowed to separate in the ice box over night. The serums were inacti-

vated at 56°C. for thirty minutes following which they were tested for antilytic power. Table 6 shows a typical protocol of several experiments.

TABLE 6

HS. Serum of rabbit immune to natural hemolysin.
AS. Serum of rabbit immune to artificial lysin.
NS. Serum of normal rabbit.
A. Supernatant fluid from a centrifugated eighteen-hour culture of *B. megatherium*.

0.5 cc. A plus 1 cc. cell suspension	++	in 8 minutes
0.5 cc. A plus 1 cc. cell suspension	++	in 8 minutes
0.5 cc. A plus 0.2 cc. HS plus 1 cc. cell suspension	−	in 1½ hours
0.5 cc. A plus 0.2 cc. HS plus 1 cc. cell suspension	−	in 1½ hours
0.5 cc. A plus 0.2 cc. AS plus 1 cc. cell suspension	−	in 1½ hours
0.5 cc. A plus 0.2 cc. AS plus 1 cc. cell suspension	−	in 1½ hours
0.5 cc. A plus 0.2 cc. NS plus 1 cc. cell suspension	++	in 60 minutes
0.5 cc. A plus 0.2 cc. NS plus 1 cc. cell suspension	++	in 60 minutes

Further experiments on the immune bodies resulting from immunization of animals with the artificial hemolysin of *B. megatherium* are given in the paper on the Nature of Toxin by Warden and ourselves.

DISCUSSION

We have dealt merely with the hemolysins of two organisms streptolysin and megatheriolysin, both of which are very powerful and occur early in the growth of the organisms. The former is only produced under special conditions and disappears in twenty-four hours. The latter is elaborated readily upon very simple media and remains active for weeks. The difference in the ease with which these hemolysins are produced appears to bear a direct relation to the rapid and abundant growth of the bacteria, the hemolysin not being present in quantities sufficient to detect until the growth is quite abundant. We are aware that with the artificial antigens we have not obtained hemolysins as active as those formed by the germs, but a discrepancy of the sort was to be expected. The microörganism delivers its antigen into the broth in a state of emulsification difficult to imitate with only the colloid properties of the broth itself, and those of the few proteins used as adjuvants to aid us in our attempts to

bring about an optimum surface for the adsorption of the antigen. The variations in the quantity of antigen necessary to produce hemolysin in the various broths used bear out the importance of the colloidal properties of the menstruum to which the artificial antigen was added, and the fact that broth that has been passed through a Berkefeld filter requires the addition of more antigen than the unfiltered broth to make it hemolytic seems also to emphasize the importance of surface in the production of artificial hemolysin.

Another point that seems at first sight to afford a distinction between the natural and artificial hemolysin is the clouding that occurs with certain doses of the antigen. This variation is however only apparent and can be avoided by emulsification of the antigen before its addition to the broth, or by the presence in the broth of just the proper surface at the time of the addition of the antigen. Clouding depends in part on the rate at which the antigen is added and upon the manner of adding it—a considerably larger amount can be introduced without formation of a cloud if the emulsification be made drop by drop slowly and with constant gentle motion.

The points in which the natural and artificial antigens resemble each other are as follows (a) both are comparably hemolytic; (b) both are inactivated by heat at approximately the same temperatures. The natural lysin of *B. megatherium* is usually inactivated by heating to 56°C. for thirty minutes, though in some specimens it was found to require 60°C. for the same length of time, while the artificial hemolysin when containing 40 to 60 mgm. of antigen per liter is inactivated at from 60° to 65°C. for one-half hour. These temperatures are sufficiently close together for the discrepancy to be accounted for by the crudeness of the artificial methods. Streptolysin was found to inactivate at 70°C. for two hours by Besredka and by Ruediger when serum medium was used and then diluted with salt solution before being passed through a Berkefeld filter. In our serum-broth medium the streptolysin was inactivated at 65°C. for one-half hour. We are forced to believe that the colloidal state of the medium has considerable effect on the temperature

at which inactivation occurs. (c) The natural and artificial lysin of the *B. megatherium* is inactivated by adsorbents under the same conditions. The natural streptolysin is not inactivated by these agents, nor is the artificial lysin in the presence of serum. (d) Analysis of the broth in which *B. megatherium* had been grown for twenty-four hours showed the presence of approximately 60 mgm. per liter of the fat complex of the organism. No analysis of the Streptococcus broth was made because of the serum present. (e) Neither natural or artificial streptolysin has thus far yielded satisfactory antilysin. Both natural and artificial lysins of the *B. megatherium* gave rise to strong specific antilysins.

We have shown that certain artificial specific fat complexes exhibit all the reactions characteristic of the bacteria examined.

We believe that the hemolysins of the organisms studied consist of the respective fat antigens of the bacteria existing in definite colloid states.

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THE NATURE OF TOXIN

THE ANTIGENS OF CORYNEBACTERIUM DIPHTHERIAE AND BACILLUS MEGATHERIUM AND THEIR RELATION TO TOXIN

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Previous work upon a considerable number of bacteria and other types of living cells having demonstrated that each variety of cell possesses a fat complex which is specifically antigenic,[1] it was determined to ascertain whether toxin-producing bacteria might not also yield fat antigen complexes, and whether such antigens bear any relationship to the toxins.

For this purpose we selected *C. diphtheriae* and the *B. megatherium*, both of which yield heavy growths as well as produce abundant toxin in suitable media. It has seemed advisable for purposes of explanation and comparison to include the observations upon both of these bacteria in one paper. The toxin of *C. diphtheriae* induces strong antitoxin, but the antigen of the organisms themselves is not conspicuous in the production of other immune substances such as agglutinins, precipitins and complement fixing bodies, while on the other hand the *B. megatherium* gives rise to abundant antibodies of such nature together with antihemolysins and antitoxins. Where the reactions of these two organisms have characters in common they tend to confirm one another, and the deficiences of one may be explained by the data obtained from the other. Moreover, the work on these bacteria was carried on at the same time, together with that of Connell and Holly on the "Nature of Hemolysins."

[1] Warden, Jour. Infect. Dis., 1918, **22**, 133; ibid., 1918, **23**, 504; ibid., 1919, **24**, 285; Warden and Connell, ibid., 1919, **25**, 399.

C. DIPHTHERIAE. THE ANTIGEN

The strain of organism used was the Park no. 8. The cultures were prepared in a manner calculated to produce the greatest luxuriance of growth and the maximum of toxin. For these purposes there was used veal infusion to which were added 2 per cent pepton and 0.5 per cent NaCl. The pepton was for the most part a "proteose" pepton prepared by the Digestive Ferments Company which was found to yield toxin of high grade. The proteose broth was adjusted to a pH of 7.9 with NaOH prior to autoclaving. The organisms were trained to rapid pellicle formation by frequent transplantations before the final inoculations upon large surfaces of the nutrient broth as recommended by Bunker[2] which were made upon shallow depths of broth in Roux flasks laid on the flat and slightly inclined, giving an area of 40 square inches per flask and a depth varying from $\frac{3}{4}$ to $1\frac{1}{2}$ inches. Good pellicles covered the surfaces in twelve hours, and the growths were maintained at 35°C. for five to six days. It was essential that the medium should be as nearly fat-free as possible, and accordingly every precaution was taken in that regard. Careful siphonage and filtration while the broth was strongly acid, that is before the addition of alkali, with strict attention to cleanliness of vessels and glassware insured a medium containing a negligible amount of fat.

At the conclusion of the incubation period the toxic broth was separated from the germ mass by filtration through paper. The germ residues were then examined for fat content by methods described in earlier articles. The total moist residue, a portion of which was kindly supplied by Dr. Clark of the laboratory of Parke, Davis and Company, used in the examination weighed over 1 kgm. The fatty acid complex obtained therefrom was found to consist of approximately 80 per cent of unsaturated acids, and 20 per cent of saturated, nonvolatile acids.

The toxin broth filtrates to the amount of 50 liters were also examined and found to contain the same fat complex in quantities averaging 60 to 80 mgm. per liter, an amount equal to about two-thirds of that obtained from the germ residue from

[2] Jour. Bact., 1919, **4**, 4.

1 liter. The addition of cresols or other similar preservatives to the cultures renders the toxin filtrates unfit for examination, and the germ residues must be freed from it before accurate examination of the fats can be made. The cresols pass unchanged into the solvents along with the fatty acids, making their purification difficult and wasteful. A trace of cresol renders an iodin determination fallacious because of the formation of additive compounds with the halogen in Hübl's solution.

Having determined approximately the composition of the fatty acid complex derived from the germ bodies and toxin broth, this complex was then tested against the serums of immunized animals—it being the custom to check the tentative analysis in this manner. Knowing the complex to contain about 80 per cent of unsaturated fatty acid corresponding closely in its various values to an oleic acid, and about 20 per cent of lower, non-volatile saturated acids having a calculated M.W. of 250, and melting point of 58°C., several trial artificial antigen complexes were prepared, having approximately the same values, from the purest obtainable fatty acids procured from sources other than the germ bodies. These artificial antigens were: No. 1, oleic acid 80 per cent, palmitic acid 16 per cent, myristic acid 4 per cent. No. 2, oleic acid 83.3 per cent, palmitic acid 16.7 per cent. No. 3, oleic acid 84 per cent, stearic acid 16 per cent. (The specimen of oleic acid used was the normal acid, having an I. V of 87.5). They were prepared for testing by combining the Na salts of the acids in the above proportions in alcoholic solution in such weights that 1 cc. contained 2 mgm. of the complex, and to each 5 parts of the solution there was added 4 parts of a 1 per cent alcoholic solution of cholesterol, the latter being employed to furnish an adsorption surface for the antigen proper.

Experiments in complement fixation were then made with the serums of rabbits that had been immunized, some with washed *C. diphtheriae* germ bodies, others with toxin. Three separate tests were made with the serums of two groups of animals, using the sheep-rabbit hemolytic system, fresh guinea-pig complement, and a control antigen suspension in salt solution of thoroughly washed diphtheria bacilli from a twenty-four hour broth

culture. The antigenic titer of the control antigen was 0.04 cc. and that of the artificial antigens 0.1 cc. of emulsions made by mixing 1 cc. of the alcoholic antigen solutions with 16.5 cc. of salt solution. Two units of amboceptor and 2 units of complement were used, and 0.5 cc. of a 2 per cent washed sheep cell suspension, all tubes being brought to a volume of 1 cc. with salt solution. The first incubation was for thirty minutes at 37°C., the second for one hour followed by standing at 20°C. for several hours. The serums of control and immunized rabbits were inactivated at 56°C. for thirty minutes. The results of the tests are shown in tables 1, 2 and 3.

TABLE 1

First group of rabbits

SERUMS	ANTIGENS				
	Control germ suspension	No. 1	No. 2	No. 3	Control anthrax antigen
1. Rabbit injected organisms	++	++	++	++	±
2. Rabbit injected organisms	++	++	++	++	+
3. Rabbit injected toxin	++	++	++	++	++
4. Rabbit injected toxin	++	++	++	++	±
5. Normal horse	−	−	−	++	−
6. Normal rabbit	−	+	−	++	++
7. Normal horse	+	+	±	++	−
8. Control, no serum	−	−	−	−	−

TABLE 2

Second test of first group, following fresh bleeding two days later

SERUMS	ANTIGENS				
	Control germ suspension	No. 1	No. 2	No. 3	Control Streptococcus antigen
1	++	+	++	−	+
2	++	+	++	±	−
3	++	+	++	++	−
4	++	+	++	++	−
5	−	±	−	−	+
6	−	−	−	+	+
7	++	−	++	++	++
Control, no serum	−	−	−	−	−

TABLE 3

Second group of rabbits, sixty days later

SERUMS	ANTIGENS			
	Control germ suspension	No. 1	No. 2	No. 3
1. Rabbit injected organisms	+	++	+	+
2. Rabbit injected organisms	+	+	+	+
3. Rabbit injected organisms	++	++	++	+
4. Rabbit injected toxin	−	−	−	−
5. Rabbit injected toxin	+	±	+	++
6. Normal rabbit	−	−	−	−
7. Control, no serum	−	−	−	−

In tables 1, 2, and 3, ++ = no hemolysis, complete inhibition; + = partial hemolysis; − = complete hemolysis, no inhibition.

From these tests it appeared that artificial antigen no. 2 gave results in closest agreement with the control diphtheria germ suspension antigen. It was accordingly employed in the succeeding work. We do not agree with the statement of A. Besson[3] to the effect that animals immunized with toxin show no immune bodies in their serums.

Agglutination and precipitation experiments were not made with these serums or this antigen, nor was any attempt made, for reasons mentioned earlier, to immunize animals with the artificial antigen, such reactions being shown better with the *B. megatherium*.

THE NATURE OF DIPHTHERIA TOXIN

The toxic broth as it comes from the filters is alkaline, having a pH varying from 8.0 to 8.33. An experiment of Connell's showed that the increased alkalinity might be due to the ammonia, of which there was found 34 mgm. against 14 mgm. in fresh broth. We had also shown that, among other changes, the broth had become richer in fats, or salts of fatty acids, and that these corresponded with those obtained from the bodies of the germs themselves.

[3] Pract. Bact., 1913, p. 269.

We assume there can be no question that diphtheria toxin is diphtheria antigen since its injection into animals produces a specific antitoxin which unites with no other known antigen. The concensus of opinion is that toxin is not a protein and does not require protein for its development.[4] If we accept, then, as one postulate the statement that toxin is specific antigen, and as another that the specific fat complexes of cells constitute their antigens, it follows that diphtheria toxin must be composed of the diphtheria fat complex in one form or another, and it should be possible to demonstrate the correctness or falsity of our assumption by means of the artificial fat antigen in a certain colloidal state.

In taking up this work we planned to commence with a broth menstruum known to yield good toxin, and to combine with it varying proportions of the fat antigen in various forms. Accordingly a standard broth identical with that used for actual toxin production was adopted. Artificial antigen no. 2 was prepared in proper proportions in the forms of the fatty acids, the alkali salts, Na and K, the ethyl esters, and the glyceryl and cholesteryl esters. The ammonium salts were too unstable for use since in presence of Na ions in excess the NH_4 ions are replaced. Cholesterol was omitted from these antigens.

We regarded the broth as a highly complex colloidal fluid containing proteoses existing as particles of varying colloidal dimensions, as shown by ultrafiltration[5] unknown organic matter from the veal infusion, coloring matter, and various electrolytes in addition to NaCl, all under the influence of a primary pH of 7.9. We assumed that the diphtheria bacilli, trained to the most rapid reproduction on the most favorable medium must also die and disintegrate rapidly according to a general principle of life and death,[6] liberating their fat complex in an emulsified or

[4] Jordan, Genl. Bact., 1918, p. 266; Guinochet, Arch. de Med. exp., 1892, **4**, 487; Hadley, Jour. Infec. Dis., 1907, Suppl. 3, p. 95.

[5] Bechhold-Bullowa, Colloids in Biol. and Med., 1919, p. 99.

[6] Vesilova, Russk. Vrach., 1915, **9**, 205; Park and Williams, Pathol. Microorg., 1920, p. 343. As an adjunct to the "life and death" principle in accounting for the death of microorganisms, in fluid cultures at a time when the quantity of lysin or toxin is at a maximum, is the fact of the existence in the fluid of the

colloidal form, which then, by reason of the necessity for such surface-tension-lowering substances to collect at the interfaces between dispersed and watery phases, become adsorbed upon colloidal particles of a certain size in the broth, forming, with electrolyte, an adsorption entity constituting toxin. We do not believe there is any evidence whatever to show that toxin exists preformed within the bodies of the bacilli and passes through their membranes into the culture medium. The particles of the toxic adsorption aggregate must be very small as we know from the ultrafiltration experiments of Bechhold[7] being slightly larger than protalbumoses and smaller than the particles in 1 per cent hemoglobin solution. We realized the impossibility of reproducing artificially all the changes undergone by medium and bacteria during the life of the culture, but we tried to imitate as closely as might be the processes of colloidal nature which we assumed on good evidence to take place.

The various methods used may be mentioned here: (a) The fatty acid antigen in varying doses, in ether solution, was overlaid on the surface of 100 cc. of broth in Erlenmeyer flasks of 250 cc. capacity. This was soon abandoned. (b) The fatty acids were floated in bulk on the broth surface. This method was also unavailable. (c) The fatty acids in alcoholic solution were pipetted upon the surface of the medium, and also emulsified by mixing at once. (d) The same methods were used with the K and Na salts, and with the ethylic, glyceryl and cholesteryl esters. Several flasks of each description were always made so as to permit of daily examination. All the operations were carried out in a sterile manner. Departures were made from the standard medium to include veal infusion without peptone, peptone solutions alone, and, finally, to eliminate all protein, colloids of mastic-fat in water and salt solution.

fatty acids, or their salts, derived from the bacteria, in just such amounts as inhibit the growth of the germs, and at the same time cause the lysis of cells. It is significant that the dose of the Na salts of the anthrax antigen just sufficient to prevent the development in broth of the bacilli from spores, 60 mgm. per liter, is that which toxic and lytic broths were found to contain, and approximately that which was added of the alkali salts to the artificial lysins and toxins, as will be shown later.

[7] Loc. cit.

The alcoholic solutions of the antigen fatty acids, salts and esters were sterile and of convenient strengths for the pipetting of small amounts to the broth with the minimum of alcohol. Control experiments showed that the addition of corresponding quantities of alcohol alone gave rise to no precipitation or other appreciable change. The antigens were added in weights varying from 1 mgm. per 100 cc. to 20 mgm. per 100 cc. in a maximum of 1 cc. of alcohol, without material change in the pH either at once or with the passage of time. After inoculation the flasks were placed, without stirring, save where emulsification was done at once, in the incubator at 35° to 37°C. where they remained undisturbed until examined. The pH of the broth was also made to vary from the standard so as to try the effects of such concentrations as 7.38, 8.0, 8.1 and 8.33. Some of the flasks were made alkaline to the lower figures by ammonia added in a sterile manner after autoclaving and cooling. Several flasks were incubated under lowered oxygen tension. When a flask was removed from the incubator the pH of the contents was taken, and the degree of clearness noted, as well as the presence or absence of sediment and of faint scum of unemulsified fat upon the surface. As a rule 2 cc. quantities taken from the center of the fluid were then injected subcutaneously into guinea pigs averaging 275 grams weight.

The results of these experiments are shown in tables 4 and 5.

TABLE 4

FATTY ACIDS	TOTAL GUINEA PIGS INJECTED	DIED
8 mg. artificial diphtheria antigen added to broth surface. Incubation at 37°C., five to six days	82	79
Same dosage K or Na salts of fatty acids added to broth surface. Incubation 37°C. five to six days	15	11
Mastic emulsions; alkali salts; no incubation	25	20

Number of pigs dying in 1 to 4 days..... 21
Number of pigs dying in 5 to 14 days..... 50
Number of pigs dying in 14 to 30 days..... 29

TABLE 5

Showing a specimen portion of the death record in greater detail

pH	7.3	7.6	7.8	7.9	8.04	8.3
Deaths	0	2	1	9	4	1
Alcoholic solution fatty acids:						
Incubation (days)	2	3	4	5	6	8–12
Deaths	1	3	1	8	2	4
Days to kill	1	2	3	4	5–10	10–14
Deaths	3	3	2	4	4	4

There were included in these tables only those animals dying without infection which presented at autopsy a definite picture of the macroscopic lesions characteristic of death from diphtheria toxin, viz., great emaciation, hemorrhages into the capsules of the adrenals and kidneys, enlarged and hemorrhagic kidneys, injection and hemorrhage of the lungs. Free fluid in the pleural sacs and intestinal injection were variable signs. The best results were obtained from dark colored broth inoculated on the surface with 8 mgm. of the fatty acid antigen and allowed to remain at 37°C. for five to six days, and which, at the time of injection was clear, or with a fine colloidal haze, had a pH of 7.9 to 8.1, a very slight or no surface pellicle and a slight sediment consisting for the most part of crystalline phosphates. Nearly all animals injected with such broth died. Filtration through a Berkefeld filter greatly diminished the toxicity. Distinctly cloudy fluids did not give good results. The antigen in the form of the K salts gave good results, while no deaths at all were obtained from the broth containing the ethyl, glyceryl or cholesteryl esters. We found that with the same broth inoculated with the same dose of antigen, in the same manner so far as control was possible, there were obtained fluids of many degrees of emulsification from clearness to dense cloudiness. The reason for this in the absence of contamination was not apparent. We had difficulty also in making different lots of broth alike, particularly in color, some being pale, others dark, depending somewhat on the quality of the veal used for making the infusion.

A notable feature of the results was the irregularity in killing time. Not infrequently a toxic broth which killed one guinea-pig in two days did not kill the other of the pair until much later. We attempted to account for this peculiar result by recognizing the extreme instability of such colloids, the slightly different conditions encountered in the tissues of the various guinea-pigs sufficing to alter the physical state of the injected fluid.

Control guinea-pigs that died following injection of Streptococcus and of Pneumococcus artificial antigen broth showed pulmonary congestion and hemorrhages without particular damage to kidneys or adrenals.

The most serviceable colloids of mastic were found, after many trials, to be those prepared by adding to an alcoholic solution of mastic of known concentration the desired amount of alcoholic solution of fat antigen, and then emulsifying in sterile water or salt solution by adding the alcoholic mixture to the fluid kept in constant whirling motion. The emulsions were pale white with orange colors by reflected light, and the particles were beyond the limits of microscopic vision. The clearest results were obtained with colloids containing 5 mgm. of mastic and 5 to 8 mgm. of the K salt antigen in 100 cc. of diluent, and brought to a pH of 7.9–8.0 with NaOH. On standing the pH shifts to the acid side. The injections were made with freshly prepared sterile emulsions.

The pathological picture exhibited in guinea-pigs dying from diphtheria and artificial toxin, while characteristic, presents certain features which in the long run do not appear to be distinctive of that poison alone. Out of the control animals in number at least equal to the determinants and kept under the same conditions, there were found two pigs dead following injections of supposedly tuberculous urine showing hemorrhages into the adrenals, and one apparently normal pig, without infection, showing the same lesion. The kidneys of these animals were not noticeably affected. These three control guinea-pigs were the only examples however, in this and in previous work upon fat antigens, in which lesions in any way similar to those of diphtheria toxin were observed.

The diphtheria fatty acid-colloidal fluids used for injection were not hemolytic for rabbit cells in the test tube, whereas those prepared with the K salts were strongly so. Vesilloff[8] showed that the bacilli from very young broth cultures removed by centrifugation and suspended in salt solution were hemolytic. Lubenau[9] states that broth cultures are hemolytic between the second and fourteenth days, varying with different strains.

In seeking an explanation for the instability of our artificial toxic colloids we were reminded of the fact that the methods of emulsification so far employed must be of the crudest nature compared with those which accompany the disintegration of the bacilli. We regard the cells, bacterial or other, as consisting of emulsion colloids of water, protein, fat, salts, etc., having at their surfaces or limiting layers an excess of those substances which lower surface tension and aid in regulating permeability, and which, according to the principle of Willard Gibbs must exist at the surfaces, namely emulsified fats, their acids and salts, and protein. The colloidal state of the limiting surfaces is probably different from that within the cells—a reversed type of colloid like a water-in-oil emulsion, in contrast to an oil-in-water emulsion to which one may compare the state of the interior of the cells.[10] Cells disintegrating or autolyzing in a watery colloidal menstruum such as broth possess dispersion means of remarkable power owing to the highly emulsified state of the fats which, liberated under such conditions, must pass to interfaces in the fluid in a manner far more delicate than we can readily approach in an artificial way. With these ideas in mind we believed we should be able to obtain greater stability in the artificial toxins if we emulsified the fat antigen prior to adding it to the broth. Accordingly we combined the antigen with solutions of the commoner proteins at hand such as hemoglobin, casein, egg albumen, gelatin, and a protein derived from the *Bact. typhosum*, substituting the alkali salts of the antigen for the fatty acids because of their somewhat greater emulsifying

[8] Russk. Vrach., 1913, October 13, p. 235.
[9] Centr. f. Bakt., 1901, **30**, 365.
[10] Clowes, Science, 1916, **43**, 750–757.

properties. The colloids formed in this manner were of great interest. If to a solution of 10 mgm. of hemoglobin in 5 cc. of salt solution there was added drop by drop the alcoholic antigen solution a somewhat opalescent colloid resulted. When this mixture was added to the standard broth in constant motion drop by drop there resulted beautifully clear, stable liquids even when the amount of fat antigen exceeded 200 mgm. per 1 liter. When, however, the hemoglobin solution alone, or the hemoglobin-antigen solution was added to the broth all at once, instead of gradually, the resulting fluids became cloudy. The same results were noted with fat-free casein-antigen and the typhoid protein-antigen mixtures. The emulsions made with fresh egg white and with gelatin were never perfectly clear.

Of particular interest were the colloids made with the typhoid protein-antigen emulsions. The typhoid protein itself, of which mention has been made in an earlier paper, is soluble in salt solution, non coagulable by heat, contains but traces of amino nitrogen and is highly toxic for laboratory animals in small doses. It is toxic also when its solution is mixed with broth by the drop method, 1 cc. of the fluid containing 0.25 mgm. injected intraperitoneally into guinea-pigs being fatal in twenty-four hours. On the other hand twice the dose produces no symptoms at all if the protein solution be added to the broth all at one time. The addition of the fat antigen to the colloid increases the toxicity, and gives to the autopsy picture its distinctive character. Guinea-pigs dying from the effects of the protein alone present no signs beyond slight injection of the visceral and parietal peritoneum whereas those dying from the protein antigen emulsions show characteristic signs of diphtheria toxin poisoning. We do not believe that the typhoid protein, derived as it is from the germ bodies that have undergone prolonged defatting extractions with alcohol and with ether, represents the proper protein of the bacteria during life, but this view does not militate against the conception that the proteins of some microörganisms liberated by autolysis in fluids may also be somewhat toxic if emulsion in a proper colloidal state occurs. We are inclined to the belief that the potentialities for toxin

production are always present when bacteria are permitted to undergo lysis in fluid culture media, and that actual toxin production depends first on the characters of the emulsified fat antigen complex and second upon its colloidal arrangement.

The mixtures of hemoglobin and broth, and of casein and broth did not show toxicity whereas these emulsions containing the fat antigen were toxic.

Still another factor instrumental in the making of suitable protein-antigen broth colloids is that of the color of the broth. The pigment of broth appears to be a distinct aid to emulsification. Very light colored broth is a much poorer colloidal medium than one which is dark. Besredka[11] noted that the filtrates of his streptococcus lysin which had lost some of the color during the filtration were impaired in hemolytic power. Connell and Holly showed that a broth which had been passed through a Berkefeld filter prior to the addition of artificial megatherium antigen had practically lost its hemolytic power as compared with the unfiltered broth containing the antigen. Very slight alterations in broth lead to great colloidal changes.

A very important factor also is the maturation of the protein-fat antigen-broth colloids. A period of time of at least one hour at 20°C. after the mixing of the ingredients is essential to the development of maximum hemolytic and toxic power, after which time, at 4°C. the activity remains stationary for a considerable period and then gradually declines. Heat inactivates these mixtures in a manner similar to true hemolysin and toxins.

The toxicity of the artificial fat-protein colloids is shown in the specimen protocol given in table 6. All the injections were

TABLE 6

Artificial colloid no. 1: 35 cc. of standard broth to which was added in divided doses 10 mgm. of typhoid protein dissolved in 5 cc. salt solution.

Artificial colloid no. 2: The same, to which the protein solution was added in a single dose.

Artificial colloid no. 3: The same, to which was added in divided doses 5 cc. of a salt solution emulsion of 10 mgm. of typhoid protein with 4.8 mgm. of K salt diphtheria antigen.

Artificial colloid No. 3A: The same, with 5 mgm. of typhoid protein.

[11] Ann. de l'Inst. Past., 1901, **15**, 880.

TABLE 6—*Continued*

Artificial colloid no. 4: 35 cc. of standard broth to which was added in divided doses 5 cc. of a salt solution emulsion containing 10 mgm. of fat-free casein and 4.8 mgm. of K salt antigen.

Artificial colloid no. 5: The same, with 10 mgm. of hemoglobin substituted for the casein.

Artificial colloid no. 6: The same, with 10 mgm. of gelatin substituted for the hemoglobin.

Artificial colloid no. 7: The same, using 100 mgm. of fresh egg white as protein.

GUINEA PIG NUMBER	AMOUNT INJECTED INTRA-PERITONEALLY	RESULTS
		Guinea-pigs injected with colloid no. 1
1	2.0	Died in 12 hours, intestines hemorrhagic
2	2.0	Died in 4 hours, intestines hemorrhagic
3	1.0	Died in 4 hours, intestines hemorrhagic
4	0.5	Lived
5	0.25	Lived
		Guinea-pigs injected with colloid no. 2
6	2.0	No symptoms
7	1.0	No symptoms
8	0.5	No symptoms
		Guinea-pigs injected with colloid no. 3
9	2.0	Died 4½ hours; typical lesions
10	2.0	Died 5 hours; typical lesions
11	2.0	Died 3½ hours; typical lesions
12	2.0	Died 7 hours; typical lesions
13	2.0	Died 6 hours; typical lesions
14	1.0	Died 7 hours; typical lesions
15	0.5	Died 15 days
16	0.5	Lived
17	1.0*	Lived
18	1.0*	Lived
19	2.0†	Lived
20	2.0†	Lived
21	2.0†	Lived

* Plus 250 units antitoxin.

† Plus 500 units antitoxin.

TABLE 6—*Concluded*

GUINEA PIG NUMBER	AMOUNT INJECTED INTRA-PERITONEALLY	RESULTS
		Guinea-pigs injected with colloid 3a
22	2.0	Died second day; typical lesions
23	2.0	Died third day; typical lesions
		Guinea-pigs injected with colloid 4
24	2.0	Died third day; typical lesions
25	2.0	Died fourth day; typical lesions
26	1.00*	Died seventh day; fair lesions
27	1.00*	Died seventh day; fair lesions
		Guinea-pigs injected with colloid 5
28	2.0	Died third day; typical lesions
29	2.0	Died first day; typical lesions
30	2.0	Died fifth day; fair lesions
31	2.0	Died tenth day; typical lesions
		Guinea-pigs injected with colloid 6
33	2.0	Died second day; good lesions
33	2.0	Lived
		Guinea-pigs injected with colloid 7
34	2.0	Lived
35	2.0	Lived

made intraperitoneally into guinea-pigs of 250 grams average weight. The autopsies showed the characteristic lesions. All the colloidal fluids were matured for one and one-half hours at 20°C.

The neutralizing action of antitoxin upon artificial toxin is indicated in the foregoing table. The dose of toxin used was one always fatal to guinea-pigs in six to eight hours. It would have occasioned no surprise had antitoxin failed to protect, since at best we had only hoped to approximate the toxic colloid in our artificial mxtures, but the experiments while they have not been

carried on to the extent one would desire seem clearly to indicate a protective influence, specific or otherwise, but proportionate to dosage, on the part of the antitoxin.

Further observations upon the production of artificial toxin mixtures have suggested the availability of emulsifying substances other than proteins, such, for instance, as the dyes, of which Congo red has thus far alone been tried. This question is of interest in connection with the coloring matter of broths noted earlier.

B. MEGATHERIUM. THE ANTIGEN

The strains of *B. megatherium* were two in number, one of our own, and that known as No. 7 kindly sent us from the Museum of Natural History, the latter having been used and commended by Rous, Robertson and Oliver.[12]

Heavy cultures of the organism were grown for twenty-four to thirty-six hours at 35°C. in Roux flasks on beef-peptone 1 per cent agar, the mass removed in small amounts of water and saponified in the manner previously described. The collected fatty acid complex was then examined and found to consist of approximately 56 per cent of insoluble unsaturated fatty acid and 44 per cent of volatile fatty acid. The saturated fraction obtained by steam distillation had a melting point of 30°C. a neutralization value of 233 mgm. NaOH, and a calculated M.W. of 175, showing that it probably consisted wholly of capric acid. The insoluble residue from the distillation, after conversion into the Pb salts and extraction with ether, showed the absence of further saturated acids, and the fluid acid obtained by conversion of the Pb salts gave an I.V. of 90, a neutralization value of 142 mgm. NaOH and a calculated M.W. of 283, data quite in agreement with an oleic acid. The tentative formula for the Megatherium antigen, then, consisted of oleic acid 56 per cent and capric acid 44 per cent. This was different from any previous complex studied but resembled in physical characters most closely that for *B. anthracis*.

[12] Jour. Exp. Med., 1919, **29**, 283.

The antigen in the form of the Na salts with cholesterol was then tested for complement fixing power with the serums of rabbits immunized with washed Megatherium organisms, and with Megatherium hemotoxin. The procedure in this experiment was identical with that used in testing the Diphtheria antigen. The results appear in table 7.

TABLE 7

SERUMS	ANTIGENS	
	Control germ suspension	Artificial fat antigen
1. Rabbit injected, organisms	++	++
2. Rabbit injected, organisms	++	++
3. Rabbit injected, toxin	++	++
4. Rabbit injected, toxin	++	++
5. Rabbit injected, toxin	++	++
6. Normal rabbit	+	+
7. Normal	−	−
8. Normal	++	++
9. Normal	−	−
Control, no serum	−	−

++ = no hemolysis, complete inhibition; + = partial hemolysis; − = complete hemolysis, no inhibition.

Table 8 shows the results of the agglutination-precipitation tests of the same serums with the artificial fat antigen.

TABLE 8

Each tube contained: Antigen solution 0.08 cc., serum 0.2 cc. and salt solution 1 cc. The tubes after mixing and shaking were placed in the ice box over night and read the following morning.

SERUM NUMBER	ACTIVE SERUM	INACTIVATED SERUM
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	−	−
7	+	+
8	−	−
Control, no serum	−	−

+ = precipitation; − = cloudy, no precipitation.

Table 9 shows the results of the precipitation test with the same serums.

TABLE 9

Antigen No. 1. True Megatherium toxin broth.

Antigen No. 2. Artificial toxin broth, composed of 40 cc. broth containing 5 mgm. of Typhoid protein and 2.4 mgm. of the K salts of the Megatherium antigen.

Each tube contained 0.5 cc. of antigen and 0.04 cc. of diluted serum. Readings taken as in table 7.

SERUM NUMBER	SERUMS DILUTED 1:60		SERUMS DILUTED 1:120	
	(1) Toxin	(2) Artificial toxin	(1) Toxin	(2) Artificial toxin
1	++	+	++	+
2	++	+	++	+
3	++	+	++	+
4	++	+	++	+
5	++	+	++	+
6	−	−	−	−
7	−	−	−	−
8	−	−	−	−
Antigen only	−	−	−	−

These experiments showed that in all probability the artificial antigen was approximately correct. We observed in these and in later tests that many normal rabbits have natural antibodies against the *B. megatherium* and its toxin. This fact was noted by Todd.[13]

In order to test further the antigenic action of the artificial fat complex, rabbits were immunized by divided S.Q. injections of 0.5 mgm. doses emulsified both in broth and in salt solution. Eight days after the sixth and last injection the rabbits were bled and the serums separated and inactivated at 56°C. for thirty minutes. The following table shows the results of complement fixation carried out in the manner previously described.

From these experiments it appeared that the serums of rabbits immunized with the artificial antigen of *B. megatherium* contained agglutinating and complement fixing antibodies in fair amount together with strong antilysins. Connell had pre-

[13] Lancet, 1901, **2**, 1663; Trans. Path. Soc. Lond., 1902, **53**, 196.

TABLE 10

SERUMS	ANTIGENS	
	Megatherium suspension in salt solution	Artificial antigen
1. Rabbit injected, salt solution emulsion	++	++
2. Rabbit injected, salt solution emulsion	++	++
3. Rabbit injected, broth emulsion	+	+
4. Rabbit injected, broth emulsion	+	+
5. Normal rabbit	±	−
6. Normal rabbit	±	−
Control, no serum	−	−

TABLE 11

The same serum agglutinated a suspension of washed Megatherium in salt solution

Each tube contained: Salt solution suspension 1 cc. and 0.04 cc. serum. Temperature 4°C. for two hours.

SERUMS	SUSPENSION
1	+
2	+
3	+++
4	++
5	−
6	−
Control, no serum	−

+++ = complete agglutination and precipitation; ++ = almost complete agglutination; + = partial agglutination; − = no agglutination.

TABLE 12

Showing the presence of antihemolysis in the same serums which were tested against the clear centrifugated lysin of a twenty-four hour veal-Bacto peptone 1 per cent broth culture of which 0.5 cc. caused complete hemolysis of 1 cc. of a 2 per cent suspension of rabbit red cells in salt solution in eight minutes at 37°C.

Each tube contained: 0.5 cc. of fresh hemolysin, 1 cc. of cell suspension and 0.04 cc. of serum. The tubes were shaken and placed in a water bath at 37°C. for two hours, and then allowed to stand at 20°C. over night.

SERUMS	0.04 CC. OF ACTIVE SERUM	0.04 CC. OF INACTIVE SERUM DILUTED 1/5 WITH SALT SOLUTION
1	−	−
2	−	−
3	−	−
4	−	−
5	++	++
6	++	++
No serum	++	

− = no hemolysis; ++ = complete hemolysis.

viously shown that the serums of rabbits immunized with Megatherium organisms and with toxin contained strong agglutinins and precipitins. The Megatherium antibodies diminish fairly rapidly in the serums of rabbits after having reached their maximum. This fact was shown by repeated experiments upon the antihemolytic power of the serums from both the series of animals immunized with germ bodies and toxin, and with the artificial antigen, they having been bled two days and again four days after the first drawing. The subsidence in antibody titer was shown to be parallel in the two series.

TABLE 13

Shows the hemolytic and toxic power of our strain of B. megatherium

Toxin no. 1: Twenty-four hour broth culture (composition given above) centrifugated clear at high speed, of which 0.04 cc. hemolyzed 1 cc. of 2 per cent red cell suspension in twenty minutes.

Toxin no. 2: Six day broth culture, centrifugated clear. 1 cc. hemolyzed 1 cc. of red cell suspension in thirty minutes.

GUINEA PIG NUMBER	AMOUNT INJECTED INTROPERITONEALLY	TOXIN NUMBER	RESULTS
1	2	1	Died in less than 12 hours
2	2	1	Died in less than 12 hours
3	2	2	Died in less than 12 hours
4	2	2	Died in less than 12 hours

Autopsies showed: Abdomen distended; peritoneum bright red, cavity containing hemolyzed blood; small intestines hemorrhagic with hemorrhages into the lumen; lungs slightly injected; bloody fluid in pleural sacs; heart muscle injected; bloody transudate over thighs.

Guinea-pigs nos. 1 and 2 showed much more intense signs than nos. 3 and 4.

This experiment indicated that both the hemolytic and toxic powers of twenty-four hour cultures of the strain were greater than those of the six day cultures and that hemolysin and toxin were probably the same substance.

DISCUSSION

We believe with Bordet,[14] Todd,[15] Craw,[16] and many others whose work our observations tend to confirm that hemolysins are true toxins. Some toxins may not be hemolytic for the reason that the toxic particle may be of a size which does not readily form adsorption aggregates with red cells, or because of the protective action of proteins or other emulsifying substances. All antigens so far examined are hemolytic in certain colloidal states.

We have brought considerable evidence to show that the toxins of *C. diphtheriae* and of *B. megatherium* probably consist of the respective fat antigens of the organisms existing in definite colloidal states, the particulate nature of the complexes being an indispensible factor. As will be stated in greater detail in another paper the particulate character of all antigens is necessary to the colloidal concept of immune processes. Just as bacteria, parasitic in the blood and tissues of an animal, are colloidal particles having specific and characteristic surface chemistry, so also are the artificial fat antigens which have been used as substitutes for the germ bodies. The mode of action of such colloids is twofold, the primary one being that of "surface," or particles, alone, the secondary one that of the specific chemistry of the particles regulating the specificity of the immune response. The injection of unorganized particulate surface (kaolin, charcoal) leads to adsorptions and induced toxicity of the plasma of the animal (anaphylaxis); injections of, or infection by, bacteria or other cells also produce adsorptions, but the character of the substances adsorbed must be different for each species of cell, depending on the chemical complex constituting its surface.

The result of such adsorptions on the body fluids is a deprivation of some of their constituents, followed by the fluids compensating, or making good their loss by an attack upon certain

[14] Bordet-Gay, Studies in Immunity, 1909, p. 186. et seq.
[15] Loc. cit.
[16] Proc. Roy. Soc. Lond., Ser. B., 1905, **76**, 179.

groups of body cells which may contain the missing substances upon their surfaces. There is considerable evidence pointing to the fact that toxins and antigens need not act directly on the cells but through the medium of the fluids bathing them. The substances primarily adsorbed, when regained gradually and in excess from the cells we regard as specific antibody.

The specific fat antigen complex of a cell may be one which in its particulate character may produce poor or ready response on the part of the body fluids, the result being inferior or strong antibody, as for instance Streptococcus and *V. cholerae;* while on the other hand the definite colloidal size of the antigen particle may be necessary to powerful antibody production, for example the *C. diphtheriae;* and again the colloidal dispersion of the antigen may be variable and still yield all antibodies from the agglutinins at one extreme to antitoxin at the other, as with the *B. megatherium.* It is conceivable also that the fluids and cells of the body respond better to some fat complexes than to others, irrespective of colloidal arrangement. At best poor antibodies result from attempted immunizations of laboratory animals with the bodies of streptococci, and the same is true with the artificial antigen and with the true streptococcus hemolysin, but considering the extraordinary colloidal richness of mammalian fluids and cells this idea does not seem so tangible as another which is, briefly, that the antigenic complexes of these microörganisms have not up to the present been employed in a proper colloidal form, and we are inclined to think that further study on the fluid media in which the bacteria are grown will throw light on the obscure problem.

A necessary corollary to these principles is that all antigen-antibody reactions, from agglutination and precipitation through complement fixation to toxin-antitoxin aggregates, are but phases of the same phenomenon acting from one extreme of the colloidal realm to the other, and that all phases must be possible with all cell antigens if only the proper colloidal state can be found. Dean[17] showed that complement fixation and precipi-

[17] Lancet, 1918, **1**, 45.

tation are phases of the same reaction, and J. Alexander[18] has seen the diphtheria toxin-antitoxin union by ultramicroscopic methods.

It will be observed that no mention has been made of the so-called "lipoids." These substances play no part in the phenomenon whatever. The term "fat" has been given a somewhat elastic use to include the fatty acids and their salts and esters. Cholesterol is not a lipoid but an alcohol. The writers are of opinion that the evidence for the existence of hard and fast lipoid substances such as lecithin and kindred bodies, as such, in the fluids and cells of the body is very unsatisfactory and doubtful. The mere fact that they may be extracted from dried tissues by certain solvents does not signify at all that they existed as entities therein. There are as many kinds of lecithin as there are kinds of tissue, and, on the other hand, Barbieri and his pupils[19] failed to find a trace of lecithin in 3000 eggs. The availability of, if not the necessity for, delicate, easily shifted, labile adsorption compounds of electrolyte-fat-protein within the body fluids is, however, undisputed, and it is probable that the whole mechanism of immunity occurs in just such emulsion colloids. The proper emulsification of bacterial and their artificial antigens with emulsifying agents is regarded as the sine qua non of toxin production. The rôle of cellular protein aside from some such action does not appear to be paramount and is not otherwise essential to antibody formation. The "type" antibody response to protein and the "specific" antibody response to cells are but phases of the same process. Fat-free protein, having no fat at its surface, has nevertheless chemical configuration and particulate size, factors assuring adsorptions and antibody production, and the antibodies respond clearly to the antigen "type" only, lacking the sharp specificity of cellular antibody for the very reason of the fat-free character of the antigen.

The similar behavior of true and artificial lysins and toxins in relation to heat, pH, reagents, adsorbents, effects on animals, etc.,

[18] Bechhold-Bullawa, footnote, p. 195.
[19] Gazzetta, 1917, **47**, 1.

has been brought out in the paper of Connell and Holly "On the Nature of Hemolysin."

We believe there has been adduced fair evidence warranting the following tentative conclusions:

1. The *C. diphtheriae* and *B. megatherium* possess characteristic fat complexes which are, under proper colloidal conditions, the true antigens of these microörganisms. Artificial fat antigens have replaced the antigens of the germ bodies in the various immune reactions.

2. The lysins and toxins of the *C. diphtheriae* and the *B. megatherium* are the same substances, being, respectively, the specific fat antigens of the microörganisms existing in definite and particular colloidal states.

3. Aside from colloidal or emulsifying activity cellular protein appears to have no place in the immune reactions studied.

THE GAS PRODUCTION OF STREPTOCOCCUS KEFIR[1]

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In her work on cheese streptococci, Miss Evans (1918) has noted the presence in Cheddar cheese of gas-forming streptococci apparently similar to a streptococcus first isolated by von Freudenreich from kefir. It was observed also that the gas, which consisted entirely of carbon dioxid, was produced much more abundantly in some media than in others. For example, in trypsin-digested milk a relatively large amount of gas was formed while in lactose broth a much smaller volume was obtained, notwithstanding the fact that the latter medium underwent a vigorous acid fermentation. This indicated that the source of the carbon dioxid might be something other than the sugar.

Aside from its purely physiological interest, knowledge of the source of the carbon dioxid produced by this organism is of scientific and practical importance in connection with the curing of Cheddar cheese. Van Slyke and Hart (1903) showed that carbon dioxid is given off from Cheddar cheese throughout the curing process. The lactose of cheese, however, is entirely consumed during the first few days; hence the source of the carbon dioxid is not the sugar. The discovery by Miss Evans of the occurrence of a gas-producing streptococcus in Cheddar cheese naturally suggested that this organism might account for the evolution of carbon dioxid from cheese of this type. In an effort to throw some light on this subject the work reported in this paper was undertaken.

[1] Published with the permission of the Secretary of Agriculture.

The two cultures employed in this work were obtained from Miss Evans and belonged to the collection used in her studies of cheese. For the determination of carbon-dioxid production the special tube designed by Eldredge and Rogers (1914) has been used. The cultures were grown in 30 cc. of broth and the carbon dioxid absorbed with barium hydroxid. Titrations were expressed in cubic centimeters of $\frac{N}{10}$ barium hydroxid neutralized.

Following the hypothesis that the carbon dioxid formed by this organism might be derived from some source other than the sugar, a number of experiments were run with various sugar-free media but in no instance was a significant amount of this gas

TABLE 1

Relation of peptone concentration to carbon-dioxid production

CULTURE	MEDIUM NO. 1: 1.0 per cent peptone, 1.0 per cent yeast, 1.0 per cent Na_2HPO_4, 0.3 per cent KH_2PO_4, 0.2 per cent lactose	MEDIUM NO. 2: Same as medium 1 except 2.0 per cent peptone	MEDIUM NO. 3: Same as medium 1 except 4.0 per cent peptone
2 ar	5.9*	5.9	5.8
2 ar	5.7	6.0	5.7
96 gq	5.8	6.0	6.0
96 gq	5.8	6.2	5.8

* Cubic centimeters of $\frac{N}{10}$ $Ba(OH)_2$ neutralized.

obtained. Other experiments were conducted in an effort to show the relation between the amount of carbon dioxid produced and the concentration of nitrogenous constituents of the culture medium. For example the evolution of carbon dioxid was measured from media consisting of 0.2 per cent lactose, 1 per cent dried yeast and varying amounts of peptone. The results of such an experiment are shown in table 1. It may be seen from this table that there was just as much carbon dioxid formed in the broth containing only 1 per cent of peptone as there was in those containing a greater concentration. The results of experiments of this type and of numerous tests with various sugar-free media of various compositions would not indicate that the gas produced by this organism is derived from the nitrogenous portion of the medium.

Organic acids naturally suggested themselves as a possible source of carbon dioxid. These were therefore tested in a broth consisting of 2 per cent of peptone, 1 per cent of dried yeast and 0.5 per cent dibasic sodium phosphate. The sodium salts of formic, acetic, propionic, butyric, caproic, lactic, malic, valeric, oxalic, tartaric, citric, and succinic acids were subjected to this test but in no case was there an increase in carbon dioxid over that obtained from the same medium without the addition of an acid.

The sugar content of the medium, of course, was considered as a possible source of carbon dioxid and experiments were con-

TABLE 2

Relation of lactose concentration to carbon-dioxid production

CULTURE	MEDIUM NO. 1 4.0 per cent peptone 1.0 per cent yeast 1.0 per cent Na_2HPO_4 0.3 per cent KH_2PO_4	MEDIUM NO. 2 Same as medium 1 plus 0.2 per cent lactose	MEDIUM NO. 3 Same as medium 1 plus 0.4 per cent lactose	MEDIUM NO. 4 Same as medium 1 plus 0.6 per cent lactose
2 ar	0.5*	7.5	13.5	22.9
2 ar	0.4	7.4	13.6	22.5
96 gq	0.4	5.8	11.7	18.8
96 gq	0.4	5.6	11.9	19.6

* Cubic centimeters of $\frac{N}{10}$ $Ba(OH)_2$ neutralized.

ducted in order to throw some light on this question. The observation of Miss Evans, that a small amount of gas is obtained in ordinary lactose broth whereas a greatly increased volume is given off by the organisms when grown in digested milk, was confirmed. Experiments conducted on this point, using a well-buffered broth and varying the lactose content showed, however, that the carbon dioxid produced increases with the increased concentration of sugar. This is true up to the point where the lactose content results in acid production beyond the amount cared for by the buffer. In table 2 are given the results of an experiment which shows the increase of carbon dioxid evolved with the increase in lactose concentration. This experiment was verified on several occasions.

From observations on this point no hesitation is felt in concluding that the source of the carbon dioxid produced in these experiments was the lactose contained in the medium and not any of the other possible sources.

It was thought, since the gas produced by this organism is apparently derived from the sugar, that the greater production of carbon dioxid in digested milk over lactose broth might be explained by the greater buffer content of the milk medium. In fact it was noted early in the work that the amount of gas obtained from lactose broth was increased with the addition of phosphate. We therefore compared in other experiments the carbon-dioxid production of these cultures in digested milk

TABLE 3

Carbon-dioxide production in digested milk and in highly buffered lactose broth

CULTURE	TRYPSIN-DIGESTED MILK	MEDIUM: Beef infusion 3.0 per cent peptone 1.0 per cent Na_2HPO_4 0.5 per cent KH_2PO_4 2.0 per cent lactose
2 ar	27.0*	23.6
2 ar	27.7	23.2
96 gq	26.5	31.1
96 gq	26.3	30.1

* Cubic centimeters of $\frac{N}{10}$ $Ba(OH)_2$ neutralized.

and in highly buffered lactose broths. The data obtained from one of these tests are given in table 3. It will be seen that the carbon-dioxid production in lactose broth may be so increased by an increase in the buffer content of the medium as to give results comparable to those obtained from digested milk.

The conclusion to be drawn from the experiments reported in this paper is that the carbon dioxid produced by organisms of the *Streptococcus kefir* type, when grown in ordinary lactose broths, is derived from the carbohydrate portion of the media. With reference to the carbon dioxid produced in the ripening of Cheddar cheese, after the original lactose content of the cheese is exhausted, it would not be safe to draw definite conclusions from these observations. However, from tests with this organ-

ism in various sugar-free media, and in media containing a variety of organic acids, it would appear doubtful whether it could be held responsible for the normal carbon-dioxid production of Cheddar cheese. With the cheese-ripening problem in view, other tests were run in which glycerol was used as a possible source of carbon dioxid. These experiments also gave negative results.

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THE IMPORTANCE OF PRESERVING THE ORIGINAL TYPES OF NEWLY DESCRIBED SPECIES OF BACTERIA

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One of the most serious difficulties with which systematic bacteriology must contend is the incompleteness of the published descriptions of new species and varieties. More care is now exercised than was formerly the case but even the most exhaustive descriptions must become incomplete as new diagnostic tests are introduced in the future. The systematists who deal with the higher plants have established the custom of preserving in museum collections the actual type specimen on which a specific description is established so that a later worker with new ideas in regard to specific characters can always examine the original plant and determine its actual characteristics.

In dealing with bacteria we cannot derive information of any special value from the study of stained slides which would correspond to the dead herbarium specimens of the botanist. The only alternative is the preservation of living cultures and this is a less satisfactory procedure in view of the fact that certain characteristics may, and sometimes do, alter as a result of long-continued cultivation on artificial media. Nevertheless the preservation of such living types of cultures offers the only possibility of stabilizing bacteriological nomenclature.

There are now at least three institutions in existence which aim to preserve type cultures for the systematic bacteriologist. Kral's Museum at Vienna (now under the direction of Dr. Pribram) has survived the war and the revolution and has just issued a new catalogue. The Museum of Living Bacteria at the American Museum of Natural History in New York has now been in operation for nearly ten years; and during the past year

a third institution, the National Collection of Type Cultures, has been established by the Medical Research Council of Great Britain at the Lister Institute under the direction of Dr. J. C. G. Ledingham.

Dr. Ledingham has asked for American assistance in his work and it is obviously most desirable that the closest coöperation should exist. He will furnish the American Museum with any new cultures he receives and we will send him all of ours that he may desire. Such an arrangement will not only make for the convenience of British and American bacteriologists, but will offer a double insurance against the loss of strains of delicate constitution.

The present note is presented to call the attention of the bacteriologists of America to the facilities offered at the Lister Institute and the American Museum and to urge upon all who may describe new bacterial species the great importance of promptly depositing with us the original type strain so that it may be available for the comparative study of systematists in future years.